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The Secretary of Health, Education, and Welfare has determined that the publication of this periodical is necessary in the transaction of the public business required by law of this Department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget through November 30, 1976.

Methotrexate (NSC-740) With Citrovorum Factor (NSC-3590) Rescue, Alone and in Combination With Cyclophosphamide (NSC-26271), in Ovarian Cancer[1]

Joseph J. Barlow* and M. Steven Piver[2]

SUMMARY

In a randomized study, 35 patients with advanced-stage epithelial ovarian cancers with progressive disease after prior single alkylating agent or combination-agent chemotherapy were treated with either intermediate high-dose methotrexate-citrovorum factor rescue alone or methotrexate-citrovorum factor rescue plus cyclophosphamide. Objective responses to both "second-line" chemotherapeutic regimens were observed. The objective response rate of 43% observed with the methotrexate-citrovorum factor plus cyclophosphamide treatment was superior to that achieved with methotrexate-citrovorum factor given alone. This combination of agents is the most effective second-line chemotherapeutic regimen for ovarian cancer that we have yet tested and we are therefore optimistic about its possibilities as first-line therapy. After early dosage modifications, serious toxic side effects were rare in this group of patients who had had extensive prior therapy.

[Cancer Treat Rep 60:527–533, 1976]

Because ovarian carcinoma is an insidious disease in a deep-seated organ with no reliable means for early diagnosis, most patients with ovarian cancer initially present with advanced disease (International Federation of Gynecology and Obstetrics [FIGO] stages III and IV). It has become increasingly apparent from recent reports (1–4) that radiotherapy has a limited role in the treatment of patients with advanced-stage ovarian cancer. Even a minimal residual abdominal tumor is rarely eradicated by radiotherapy alone (1,5), and in women with palpable residual tumors better therapeutic results have been achieved by chemotherapy alone (6). Even the adjunctive role of radiotherapy in the treatment of patients with FIGO stages I and II ovarian carcinoma is in question when compared with adjunctive chemotherapy (7). Consequently, chemotherapeutic agents are being employed with increasing frequency as the initial mode of therapy in patients with advanced stages of ovarian cancer, for recurrence, or as an adjunct to surgery in patients with stage I and II disease.

Until reliable means for the early diagnosis of ovarian cancer are available, most patients will enter for treatment with advanced-stage disease and will be candidates for primary chemotherapy after surgery. Although the initial objective response rates in women with advanced-stage epithelial ovarian carcinoma average almost 50% (8), the incidence of relapse after initial response is high and the overall 5-year survival rate is < 10%. It is therefore apparent that not only are more effective initial chemotherapeutic regimens necessary, but also that 90% of patients who have already begun receiving chemotherapy may require an attempt with "second-line" chemotherapy after an initial chemotherapeutic failure or relapse.

We present our experience with intermediate high-dose methotrexate (MTX) with citrovorum factor (CF) "rescue" versus intermediate high-dose MTX-CF plus cyclophosphamide (CTX) as second-line chemotherapy in patients with advanced-stage epithelial ovarian cancers who had failed or relapsed after treatment with a single alkylating agent and/or combination chemotherapy.

PATIENTS AND METHODS

Thirty-five patients were entered into the study (table 1). All patients had histologically proven advanced-stage epithelial ovarian cancer which was serous, mucinous, endometrioid, mesonephric, or un-

[1]Received Dec 3, 1975; revised Mar 5, 1976; accepted Mar 10, 1976.

[2]Department of Gynecology, Roswell Park Memorial Institute, Buffalo, NY.

*Reprint requests to: Joseph J. Barlow, MD, Department of Gynecology, Roswell Park Memorial Institute, 666 Elm St, Buffalo, NY 14263.

differentiated adenocarcinoma. All patients had objective evidence of tumor progression while receiving chemotherapy which immediately preceded their entrance into the study. In some patients, tumor progression represented a failure of their initial chemotherapeutic regimen, whereas in others, tumor progression occurred after an initial response to prior chemotherapy. Only six patients had had prior pelvic and/or whole abdominal external-beam irradiation which they had had 10 or more months prior to the study.

When entered into the study, all patients were stratified as to whether their tumor progression had occurred on single-agent or multiple-agent chemotherapy and they were randomly allocated to receive either MTX-CF alone or MTX-CF plus CTX. Most of the patients entered into the study had been receiving melphalan as single-agent therapy or actinomycin D, 5-fluorouracil (5-FU), and CTX as combination therapy. Prior therapy with melphalan was given at a dosage of 0.2 mg/kg/day in divided doses for 5 days for a total dose of 1 mg/kg/5-day cycle. The 5-day cycles were repeated every 4 weeks if white blood cell (wbc) and platelet counts were adequate. The treatment regimen with actinomycin D, 5-FU, and CTX consisted of 0.5 mg of actinomycin D, 8 mg/kg of 5-FU, and 7 mg/kg of CTX. All three agents were given iv for 5 days for a total dose of 2.5 mg of actinomycin D, 40 mg/kg of 5-FU, and 35 mg/kg of CTX. The 5-day cycle was repeated every 4 weeks if the blood cell counts had returned to satisfactory levels. Four patients randomized to the MTX-CF arm of this study had received both single-agent and combination chemotherapy before entrance into the study (table 1). Three patients randomized to MTX-CF plus CTX therapy failed to respond to both single and combination therapy before entering into the study. No patient in the study had received prior MTX in conventional dosage either alone or as a part of combination therapy.

TABLE 1.—Patient characteristics

Parameter	Treatment group	
	MTX-CF	MTX-CF + CTX
Total No. of patients	17	18
Age (yrs)		
Mean	55.8	50.7
Median	56	50.5
Range	35–75	39–60
Disease duration from diagnosis to MTX-CF therapy (mos)		
Mean	17.7	17.4
Median	14	11
Range	2–60	3–78
No. of patients with—		
FIGO disease stage		
III	8	8
IV	9	10
Histologic tumor type		
Serous	12	10
Mucinous	1	2
Endometrioid	1	2
Mesonephric	0	1
Unclassified	3	3
Histologic differentiation		
Well	1	1
Medium	8	11
Poor	8	6
Prior chemotherapy		
Single agent	7(7.9)*	8(7.6)
Combination	6(8.5)	7(10.6)
Both	4(4.7)	3(5.3)
Prior radiotherapy		
Whole abdominal	1	2
Pelvic	1	2

*Mean No. of courses or mos.

MTX was given iv as a 4-hour infusion to all patients, whether administered alone or in combination with CTX. Exactly 6 hours after the completion of the MTX infusion "CF rescue" was begun. CF was always given im at a dose of 10 mg/m². Those patients who were randomized to the MTX-CF plus CTX combination received the MTX-CF exactly as described and, in addition, received CTX iv daily for 5 days beginning on the day of the MTX infusion.

At the outset of the study the starting dose of MTX was 300 mg/m² with escalation in the absence of response or significant toxicity. Shortly after the study began it was apparent that there were few responders at the 300-mg/m² dosage level and patients were subsequently usually given 450–500 mg/m² of MTX. No patient received > 1000 mg/m² of MTX/course. Also, at the beginning of the study CF was given im in the above dosage every 6 hours for a total of eight doses. Toxicity observed early in the study led us to extend the administration of CF to 12 doses. The initial dosage of CTX in combination with MTX-CF was 300 mg/m²/day for 5 days. Because of this early excessive toxicity, the dosage of CTX was decreased to 250 mg/m²/day for 5 days. The study was arranged so that the dose of CTX in combination with MTX-CF would be equivalent to the amount of CTX in the actinomycin D, 5-FU, and CTX combination.

Hydration with 3000 ml of fluid was initiated just prior to therapy and was given daily for 4 days. This ensured a daily urine output in excess of 2000 ml. Alkalinization of the urine was not carried out.

All but one of the patients had a serum creatinine of 1.2 mg/100 ml or less and/or a creatinine clearance of 75% or more of normal before entering the study. The one patient who failed to meet these criteria received a decreased dose of MTX. No patient entered into the study had historical or chemical evidence of significant parenchymal hepatic disease.

DEFINITION OF TUMOR RESPONSE

Complete remission was defined as a complete disappearance of all measurable disease and the development of no new lesions. Partial remission was defined as a > 50% reduction in the product of perpendicular tumor diameters and/or the complete control of malignant effusions. Improvement was defined as a > 25% but < 50% reduction in the product of perpendicular tumor diameters and/or partial control of malignant pleural effusions. Stationary disease was defined as the absence of growth of measurable disease or a change in the malignant effusions during therapy only if preceded by and/or followed by definite tumor progression. Progression was defined as a 25% or more increase in measurable disease, an increase in malignant effusions, and/or the appearance of new lesions. All of the above response criteria must have been present for a period of 1 month or more.

RESULTS

Group Comparability

Table 1 compares the patient characteristics of the MTX-CF and MTX-CF plus CTX groups with respect to the number of patients entered into each group, age, disease stage, histologic type of epithelial ovarian cancer, histologic grading of the tumor, duration of disease, and prior therapy. Even though the MTX-CF plus CTX group tended to be somewhat younger, the two patient groups were quite comparable.

Therapeutic Response

A summary of the patient responses to either MTX-CF alone or in combination with CTX is shown in table 2. The patients are divided into groups according to the dosage of MTX used and whether they received MTX-CF or MTX-CF plus CTX. The dose of CTX remained constant for all patients. Both the total and the evaluable number of patients are listed for each of the groups. Patients were considered inevaluable for response if they were not re-examined at least once 1 month after the completion of a course of therapy. Twenty-nine patients were evaluable, 13 in the MTX-CF alone group and 16 in the combination MTX-CF with CTX group.

One partial response was observed with a dose of 300 mg/m² of MTX-CF plus CTX, whereas no objective responses were seen with a 300-mg/m² dose of MTX-CF alone. In the 450–1000-mg/m² dosage range of MTX there was a significantly greater objective response rate with the combination therapy compared with MTX-CF alone. There was a 46% evaluable partial response rate (43% absolute) in those patients treated with combination therapy compared with an 18% evaluable partial response rate (13% absolute) in those patients receiving MTX-CF alone. In addition, two of the patients receiving combination therapy had improvement. There were no complete responses to therapy.

A detailed summary of those patients who had objective responses to therapy is shown in table 3. The first two patients (Nos. 1 and 2) listed in table 3 responded to MTX-CF alone. The remaining seven patients (Nos. 3–9) responded to the combination of MTX-CF plus CTX. All patients had measurable abdominal and/or pelvic masses and most also had malignant effusions. The dosage and number of courses of treatment of present therapy are listed according to the dose of MTX. Of the nine responders, five had shown tumor progression during prior combination chemotherapy and four had progressed while receiving single-agent therapy. Three of the seven patients responding to the combination

TABLE 2.—Response of ovarian cancer to MTX-CF alone or with CTX

MTX (mg/m²)	Total No. of patients/ No. evaluable	No. of patients with—					% total response	
		Complete response	Partial response	Improvement	Stationary disease	Progressive disease	Evaluable	Absolute
300	2/2	—	—	—	1	1	—	—
300 + CTX	4/3	—	1	—	—	2	33	25
450–1000	15/11	—	2	—	3	6	18	13
450–1000 + CTX	14/13	—	6	2	3	2	46	43
Totals	35/29							

TABLE 3.—Objective responses to MTX-CF alone or with CTX

Patient No.*	Age (yrs)	Histologic type and stage	Microscopic grade (differentiation)	Measurable disease	Disease duration (mos)	Previous therapy (No. of courses or mos)	Present therapy with MTX		Response		Status
							Mg/m² × courses	Total No. of courses	Type	Duration (mos)	
1	57	Mucinous, stage III	Well	Abdominal and pelvic masses + ascites	35	Actinomycin D, 5-FU, CTX (22)	300 × 1 700 × 8 1000 × 2	11	Partial	10	Alive, residual disease, still receiving chemotherapy
2	64	Serous, stage IV	Medium	Pelvic mass and effusions	4	Actinomycin D, 5-FU, CTX (2)	1000 × 1 750 × 2	3	Partial	2	Dead
3	44	Unclassified, stage IV	Poor	Abdominal mass and effusions	11	XRT,† whole abdomen; adriamycin + CTX (7), vincristine + CTX (3)	500 + CTX × 2 750 + CTX × 2	4	Partial	4	Alive, progressing
4	47	Serous, stage III	Poor	Abdominal mass and ascites	8	Actinomycin D, 5-FU, CTX (7)	450 + CTX × 3	3	Partial	2	Probable drug-related death
5	49	Serous, stage III	Medium	Pelvic mass and ascites	6	CTX (2), melphalan (3)	500 + CTX × 2	2	Partial	1	Dead
6	57	Endometrioid, stage IV	Medium	Abdominal and pelvic masses + effusions	78	Melphalan (15)	300 + CTX × 2 500 + CTX × 1	3	Partial	2	Dead
7	60	Serous, stage IV	Medium	Liver and bone	10	Melphalan (7)	250 + CTX × 3 250 × 1	4	Partial	2	Progressive disease
8	50	Unclassified, stage III	Poor	Abdominal mass	15	XRT, whole abdomen; melphalan (8)	500 + CTX × 5	5	Partial	4	Dead
9	57	Endometrioid, stage IV	Medium	Abdominal mass and effusions	56	XRT, pelvis; 5-FU + melphalan (15)	450 + CTX × 5 300 + CTX × 1	6	Partial	6	Alive, changed to oral chemotherapy

*Patients 1 and 2 had MTX-CF alone; patients 3–9 had MTX-CF + CTX.
†XRT = external beam radiation.

TABLE 4.—Toxic effects of MTX-CF alone or with CTX

MTX (mg/m²)	Total No. of courses/No. evaluable	No. of courses with— No toxic effects	Oral ulcerations	Severe leukopenia (<1000)*	Severe thrombocytopenia (<50,000)*	Drug-related deaths
700–1000	21/19	18	1	1	1	0
700–1000 + CTX	10/10	8	1	1	1	1
450–500	16/15	14	1	1	1	1
450–500 + CTX	27/25	20	2	5	3	1
300	5/ 5	5	—	—	—	—
250–300 + CTX	12/12	9	—	3	2	2
Totals	91/86	74	5	11	8	5

*Cells/mm³.

MTX-CF plus CTX had previously received CTX, either alone or as part of combination therapy. Patient No. 3 had received 700 mg of CTX iv in combination with both adriamycin (70 mg/m²) and vincristine (1 mg) every 3 weeks. Patient No. 5 had received oral CTX (50 mg) three times/day until toxicity. Patient No. 9 had received the combination of 5-FU and oral melphalan. Melphalan was given in the dosage previously described and 5-FU was given at a dose of 15 mg/kg iv every day for 5 days. The courses of melphalan and 5-FU were repeated at 4-week intervals. One patient (No. 7) had a prolonged period of moderate thrombocytopenia after her third course of MTX-CF plus CTX and was therefore given only MTX-CF as her fourth course of therapy. Two of the responding patients had received prior whole abdominal radiotherapy and one had received pelvic radiotherapy.

There were three patients who progressed after receiving MTX-CF and were crossed-over to treatment with MTX-CF plus CTX. Two of these patients continued to progress and the other has had stabilization of her disease after two courses of MTX-CF plus CTX.

TOXICITY

The 35 patients in this study received a total of 91 courses of treatment with either MTX-CF alone or in combination with CTX. Eighty-six of the 91 courses of therapy were evaluable for toxicity. Seventy-four of 86 evaluable courses showed no significant toxicity. Table 4 lists the serious toxic manifestations according to whether the patient received MTX-CF alone or in combination with CTX and according to the dose range of MTX used. Eleven courses of therapy were associated with severe toxic side effects, chiefly leukopenia with or without thrombocytopenia and mucositis. Serious side effects were noted at all dose levels of MTX-CF plus CTX, but no toxicity was seen with 300 mg/m² of MTX-CF alone. Five patients (14%) were considered to have died as a consequence of chemotherapy. Two of the five were hospitalized after their last course of chemotherapy and were known to have died from chemotherapeutic complications. Three of the five died within 3 weeks of their last course of chemotherapy and were assumed to be chemotherapeutic deaths although no definitive details are available.

All of the chemotherapeutic deaths occurred before we extended the period of CF rescue and before we decreased the daily dosage of CTX to 250 mg/m². Four of the five chemotherapeutic deaths were in patients who received the combination of MTX-CF plus CTX. Since the CF rescue has been extended to 72 hours there have been no toxic manifestations attributable to MTX. Also, since dose modifications of CF and CTX have been made there has been only one severe leukopenic response with the combination of MTX-CF plus CTX. We feel that the high degree of toxicity seen early in the study was a reflection of the relatively short period of CF rescue and the dosage of CTX in a group of patients who were approaching the terminal stages of their disease after intensive and/or prolonged prior chemotherapy.

No progressive nephrotoxicity or hepatotoxicity was observed in this study. Nausea, vomiting, and diarrhea were almost never seen with the MTX-CF alone. Mild nausea and vomiting were not uncommon when CTX was added.

Plasma MTX levels[3] were performed in nearly one half of the patients at 48 and 72 hours after initiation of the MTX infusion (9). These measurements were initiated after we had extended the period of CF rescue to 72 hours. No toxicity was seen, and in

[3]Kindly performed by Dr. Jaw-Jiang Wang.

only two courses, in different patients, were the 72-hour MTX levels $> 1 \times 10^{-7}$ M.

DISCUSSION

Despite a relatively good initial response rate of epithelial ovarian cancer to single alkylating agents and to a number of combination chemotherapeutic regimens, 90% of all patients to whom chemotherapy is given will require second-line chemotherapy because of a failure to respond or relapse. This group includes not only those who enter treatment with advanced-stage disease, but also the 20%–50% of women with localized disease who relapse after surgery with or without additional radiotherapy. The reported response to second-line chemotherapy has, in general, been very poor. The most optimistic report is that of Smith and Rutledge (10) who have reported a 39% objective response rate with actinomycin D, 5-FU, and CTX in 47 patients who had failed prior single-agent chemotherapy. We have been unable to confirm their second-line results with actinomycin D, 5-FU, and CTX. We have used the combination as second-line chemotherapy in 21 patients who have either failed or relapsed after melphalan therapy and in 11 patients who have either failed or relapsed after other single-agent or combination-agent regimens. We have thus far seen *no* objective second-line responses to the combination. Thirteen of these three-drug combination failures have been previously reported (4). Poor results to second-line chemotherapy have also been reported with adriamycin (11–14) and high-dose CTX (15).

In view of our disappointing past experience with second-line chemotherapy in epithelial ovarian cancers, we are particularly encouraged by the results of the present study. Intermediate high-dose MTX-CF plus CTX gave a 46% (43% absolute) objective response rate. Several of the responses were of a meaningful duration. All patients were progressing on prior chemotherapy and were rapidly approaching the terminal stage of their disease. Also, considering the patients' disease status and the intensity and duration of prior therapy, the combination of MTX-CF plus CTX was well tolerated. The combination was particularly well tolerated with minimal toxic manifestations after the CF rescue was extended to 72 hours and the daily dose of CTX was reduced to 250 mg/m^2. We did not give > 1000 mg/m^2 of MTX because of our concern about a "third-space effect" of the malignant effusions which most of these patients had (16,17). We do feel that many of the patients could have tolerated higher doses of MTX, and in one patient (No. 1) there appeared to be an improved response with dose escalation. After an initial response to 300 mg/m^2 of MTX which was maintained through eight doses of MTX at 700 mg/m^2, she began to progress slightly. However, this tendency to progress was reversed by increasing the dose of MTX to 1000 mg/m^2.

It is apparent from this study that there was no absolute cross resistance between MTX-CF or MTX-CF plus CTX and the other prior chemotherapy which had been given to these patients. Several lines of reasoning suggest that MTX-CF and CTX act synergistically: (*a*) the combination MTX-CF plus CTX was found to be superior to MTX-CF alone; (*b*) three of seven patients responding to MTX-CF plus CTX had received prior CTX in adequate or equivalent dosage; (*c*) we had previously had poor results with high-dose CTX in women who had failed to respond or who had relapsed after prior alkylating-agent chemotherapy (15); and (*d*) it is unusual to observe a response to one alkylating agent after failure with a prior alkylating agent.

Conventional doses of MTX alone (18) or in combination (19) have yielded response rates of 25% (four of 16 patients) and 63%, respectively. A subsequent study by Greenspan and Bruckner (20) comparing MTX and thioTEPA indicated that MTX was inferior to thioTEPA alone. We know of no prior report utilizing high-dose MTX-CF in women with ovarian cancer. An advantage of MTX-CF over standard doses of MTX is its reduced toxicity which allows for combinations with full dosage of other agents.

Because agents found to be effective as second-line chemotherapy have usually proved to be even more effective as first-line therapy, we are optimistic about the possibilities of MTX-CF plus CTX as primary chemotherapy in epithelial ovarian cancer.

LIST OF COMPOUNDS

Actinomycin D: NSC-3053
Adriamycin: NSC-123127; CAS reg. No. 23214-92-8
Citrovorum factor: NSC-3590; CAS reg. No. 6055-86-5; glutamic acid, *N*-[*p*-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl]-, calcium salt
Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate
5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8
Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-
Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-
Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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Chemotherapy Versus Chemoimmunotherapy of Head and Neck Cancer: Report of a Randomized Study[1,2]

S. P. Richman,* R. B. Livingston, J. U. Gutterman, J. Y. Suen,[3] and E. M. Hersh[4,5]

SUMMARY

Thirty-four patients with recurrent squamous cell carcinoma of the head and neck were randomized to receive the five-drug chemotherapy regimen BACON (bleomycin, adriamycin, CCNU, vincristine [Oncovin], and mechlorethamine [nitrogen mustard]; 14 patients) or the same regimen plus bacillus Calmette-Guérin (BCG) by scarification (20 patients). The majority of both patient groups had received prior surgery and radiation. The patients treated with BACON plus BCG experienced a significantly longer survival ($P = 0.014$) than those treated with BACON alone. There were five drug-related deaths, and eight other patients required hospitalization for treatment of drug-related morbidity.

[Cancer Treat Rep 60:535–539, 1976]

Systemic chemotherapy of advanced epidermoid carcinoma of the head and neck has had limited success. A large number of agents have shown activity including methotrexate (1,2), bleomycin (3), cyclophosphamide (1), mechlorethamine (1), 5-fluorouracil (1), hydroxyurea (1), CCNU (4), and adriamycin (5). However, response durations have usually been brief, ranging from 1 to 5 months (1). This may relate to the fact that patients usually have had extensive prior surgery and radiotherapy and are often malnourished (6) and immunologically incompetent (7,8).

In an attempt to increase and prolong responses, we designed a combination chemoimmunotherapy protocol for patients with advanced epidermoid carcinoma of the head and neck who had failed or could not be helped by additional surgery or radiotherapy. The chemotherapy protocol was given the acronym BACON (bleomycin, adriamycin, CCNU, vincristine [Oncovin], and mechlorethamine [nitrogen mustard]).[6] It was designed to combine three active and unrelated myelosuppressive agents with the minimally myelosuppressive but active combination of vincristine and bleomycin (9).

In addition to chemotherapy, the patients were randomized to receive or not to receive bacillus Calmette-Guérin (BCG) by scarification. BCG has been shown to augment established delayed-type hypersensitivity (DTH) to recall antigens in cancer patients (10). It can prolong the disease-free interval and survival after surgery for recurrent metastatic malignant melanoma (11–13) and can prolong chemotherapy-induced remission and survival in acute myelogenous leukemia (14,15). In addition, when combined with chemotherapy BCG can increase the remission rate and can prolong the duration of

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[3]*Present address*: Department of Otolaryngology, The University of Arkansas Medical Center, Little Rock.

[4]Departments of Developmental Therapeutics and Surgery, The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston.

[5]We wish to thank Pam Roper and Amzie Miller for skin testing the patients; K. Dandridge, S. Livingston, and C. Murray for administering BCG to the patients; and Tonya Smith for assistance with statistical computations.

**Reprint requests to*: Stephen P. Richman, MD, Department of Developmental Therapeutics, The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, 6723 Bertner Ave, Houston, Tex 77025.

[6]Bleomycin: NSC-125066; [2,4′-bithiazole]-4-carboxylic acid, 2′-(2-aminoethyl)-, monohydrate.

Adriamycin: NSC-123127; CAS reg. No. 23214-92-8.

CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-.

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1).

Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2′-dichloro-*N*-methyl-, hydrochloride.

stability, remission, and survival in advanced malignant melanoma (16). It was hoped that BCG would have a similar action in this patient group.

PATIENTS AND METHODS

Thirty-four patients with histologically documented metastatic or locally recurrent squamous carcinoma of the head and neck were treated. They were eligible for this study if their disease could no longer be controlled by further surgery or radiotherapy and if they had not received prior chemotherapy with agents in this study or with methotrexate. Patients were randomized to receive BACON alone (14 patients) or BACON plus BCG (20 patients). All eligible patients were accepted and randomized. Written informed consent was obtained from all patients. Unless already hospitalized, the patients were treated in the outpatient clinic.

The therapeutic regimen is shown in table 1. The CCNU was initially administered every 4 weeks, but prolonged and significant myelosuppression resulted in a change to every 8 weeks. BCG was given as lyophilized Pasteur strain, 6×10^6 organisms by scarification on the upper arms and legs as previously described (12). Scarification was timed to be given weekly between chemotherapy. This was felt to be as often as was practical while avoiding administration at the time of chemotherapy-induced immunosuppression.

Lesions were measured as the product of their two greatest perpendicular diameters. A CR was defined as disappearance of all measurable disease. A PR was defined as 50% or greater regression of all measurable disease without the development of new disease but without a CR. Response duration was calculated from the start of response. Survival was calculated from the start of this regimen.

Eighteen of 20 patients treated with BACON and BCG were skin tested prior to therapy with five DTH recall antigens (dermatophytin, dermatophytin-O, streptokinase-streptodornase, mumps, and *Candida*), intermediate-strength purified protein derivative (PPD), and keyhole limpet hemocyanin (KLH) as previously described (17). Fourteen of the 20 were retested 1 month later. Patients treated with BACON alone were not tested.

The statistical method used was the Wilcoxon test with a two-tailed analysis for evaluating differences between remission and survival curves (18).

TABLE 1.—Therapeutic regimens: BACON alone and BACON + BCG

Drug	Dosage
Starting on Day 1:	
CCNU	65 mg/m² orally q8wks
Mechlorethamine	8 mg/m² iv push q4wks
Adriamycin	40 mg/m² iv q4wks (dose limit = 450 mg/m²)
Starting on Day 2, then weekly for 6 doses:	
Vincristine	1.0 mg (patients < 50 yrs) and 0.75 mg (patients > 50 yrs) iv push in AM
Bleomycin	30 mg iv 6–12 hrs later
± BCG	6×10^6 organisms by scarification, Days 7,14,21 of each course

RESULTS

Retrospective Comparison

The anatomic diagnosis, sex, and age distributions are shown in table 2. The two groups of patients were retrospectively examined for several prognostically important factors to test their comparability (table 3). Performance status, which has been related to median survival in bronchogenic carcinoma (19), was not statistically different in the two groups. The absolute lymphocyte counts prior to chemotherapy, another prognostic factor in cancer (20), were also not significantly different. The time from diagnosis of primary tumor to the first recurrence and the total period from diagnosis of primary to the start of this protocol were greater for the BACON plus BCG group, but these differences were not statistically significant. Both groups of patients received approximately the same medical attention as measured by the number of outpatient visits per month during the study. A majority of both groups

TABLE 2.—Characteristics of patient population

Clinical parameter	BACON	BACON + BCG
Diagnoses		
Tongue	2 (14%)	2 (10%)
Oral, oropharynx, tonsil	3 (21%)	8 (40%)
Nasopharynx	3 (21%)	3 (15%)
Skin, lip	1 (7%)	2 (10%)
Larynx, hypopharynx, pyriform sinus, epiglottis	4 (30%)	4 (20%)
Nasal sinus	1 (7%)	1 (5%)
	14	20
Sex		
Male	10 (71%)	14 (70%)
Female	4 (29%)	6 (30%)
Age (median and range)	58.5 (27–79)	61.5 (26–76)

TABLE 3.—BACON vs BACON + BCG: retrospective comparison of possible prognostic factors

	BACON	BACON + BCG
Karnofsky performance status	6.0 ± 1.8*	6.5 ± 0.94
Baseline absolute lymphocyte count (cells × 10^3)	1.0 ± 1.0	1.1 ± 0.63
No. of patient clinic visits/mo	2.1 ± 1.3	2.4 ± 2.1
Time from diagnosis of primary to—		
First recurrence (mos)	7.2 ± 9.1	16.0 ± 19.6
Start of chemotherapy (mos)	13.6 ± 15.0	24.0 ± 28.0
Prior surgery (other than biopsy)	71%	95%
Prior radiotherapy	93%	85%

*All means are not significantly different.

had undergone surgery and radiotherapy prior to the initiation of this protocol. The site of the recurrent tumor being treated was compared for the two groups. In the BACON-alone group, 43% of the treated tumors represented local recurrences in the head and neck and 57% were distant metastases, usually in the lung. These figures for the BACON plus BCG group were 55% and 45% respectively.

Therapeutic Results

The overall response rate was 44% (table 4). The response rates for the two treatment groups were not significantly different. However, it is of note that all three CRs were in the BACON plus BCG group and that all three were in patients with nasopharyngeal carcinoma. Although the number of patients with this diagnosis was small, the high overall response rate (83%, table 5) for all six patients with this diagnosis is of note. Although the median duration of response for the BACON plus BCG group (19.5 weeks) was longer than for the BACON-alone group (14 weeks), this difference was not statistically significant. The crucial difference (fig 1) is the significantly longer survival ($P = 0.014$) of the patients treated with BACON plus BCG compared to patients treated with BCG alone. The median survival was 21 weeks for both groups combined, 30.5 weeks for the BACON plus BCG group, and 13.5 weeks for the BACON-alone group. Of the 15 patients responding in both treatment groups combined, six were being treated for local recurrences and nine for distant metastases. Five deaths occurred in the first 8 weeks, three due to infection, one due to progressive disease, and one of unknown cause. Four of these deaths occurred in the patients treated with BACON alone.

Immunologic Data

Adequate baseline and followup data were obtained only for the BACON plus BCG patients. Nine of the 18 patients tested at baseline were immunoincompetent, arbitrarily defined as less than two positive reactions to the five recall antigens. Fourteen of the 20 BACON plus BCG patients had followup DTH skin tests approximately 1 month after baseline data were collected. Mean values for *Candida*, PPD, and KLH increased significantly. There

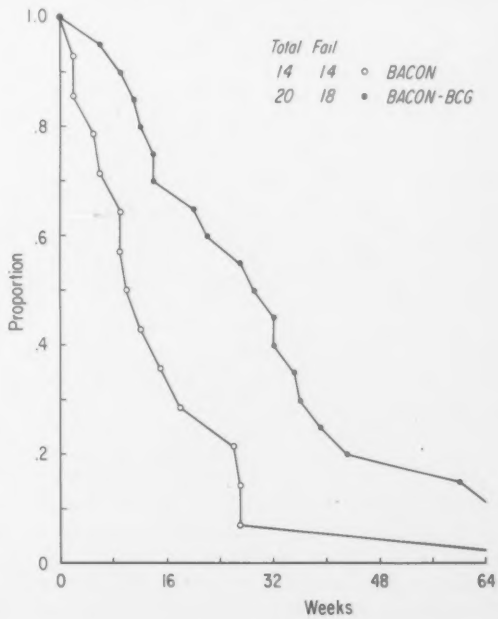

FIGURE 1.—Survival of patients: BACON alone vs BACON plus BCG. Difference in survival, $P = 0.014$, 2-tailed test.

were no statistically significant correlations between response or survival and improvement in DTH to recall antigens, PPD, or KLH. There was no correlation between a positive baseline PPD reaction and clinical response.

TABLE 4.—BACON vs BACON + BCG: response by regimen

	BACON	BACON + BCG	Total
No. of—			
CR	0	3	3
PR	7	5	12
No response	7	12	19
	—	—	—
	14	20	34
% CR + PR	50	40	44

TABLE 5.—Response by site (all patients)

Site	No. of responders/ No. in group	%
Tongue	2/4	50
Oral, oropharynx, tonsil	3/11	27
Nasopharynx	5/6	83
Skin, lip	1/3	33
Larynx, hypopharynx, pyriform sinus, epiglottis	3/8	37
Nasal sinus	1/2	50

Toxicity

Toxicity was significant, reflecting the intensity of the regimen and the already debilitated condition of the patient population. Five of 34 patients died of drug-related causes, four of infection and one of bleomycin pulmonary toxicity. Eleven other patients were hospitalized during the regimen, eight for treatment of infection, two for blood and platelet transfusions, and one for treatment of probable bleomycin pulmonary toxicity. Bone marrow suppression was the dose-limiting toxicity. There was a cumulative effect on the granulocyte and platelet counts although this did not occur frequently enough to affect the median (table 6). The immunotherapy did not result in noteworthy toxicity. Of the five drug-related deaths, four occurred in the group treated with BACON alone, and of the eight hospitalizations for infection, five occurred in the BACON plus BCG group.

TABLE 6.—Myelosuppression due to BACON and BACON + BCG (all patients)

	1st course	Subsequent courses
Median lowest granulocyte count $\times 10^3$	1.0	0.9
Median lowest platelet count $\times 10^3$	157	128
% of courses with granulocytes $\leq$ 500 cells	25	36
% of courses with platelets $\leq$ 50,000 cells	4.0	36

DISCUSSION

The improved survival of the cancer patients receiving immunotherapy is a phenomenon that has been seen in other settings cited in the introduction. A review of a number of prognostic factors for these two groups of patients indicated that they were quite comparable. However, the group of patients treated with BACON alone may have had an inherently worse prognosis based on a shorter mean time from diagnosis of primary disease to first recurrence and from diagnosis of primary to initiation of chemotherapy, possibly indicating more aggressive disease. Four of the five early deaths (< 8 weeks from the start of therapy) occurred in this patient group.

Patients with squamous carcinoma of the head and neck are frequently immunoincompetent and may remain so even after cure by radiotherapy (21). It is therefore of note that the patients treated with BCG in this study experienced a significant increase in DTH to *Candida*, PPD, and KLH skin tests which was probably due in part to the BCG, although this cannot be proved in the absence of the appropriate control.

It is interesting that the three CRs and a high overall response rate were seen in the six patients with nasopharyngeal carcinoma. Special immunologic mechanisms may be involved here since this tumor is infiltrated with lymphocytes and is associated with an elevated antibody titer to Epstein-Barr virus (22). In the future response of these tumors to therapy could be correlated with evidence of antiviral immunity. If such a correlation were found, manipulation of antiviral immunity could be considered.

Differences in patient populations and methods of evaluation make comparison of studies difficult. However, it is fair to say that the 44% response rate in this study falls in the vicinity of the results with methotrexate (1). Leone et al (23) have reported some of the best results with methotrexate with a

57% response rate and 31% CRs. Median duration of response was 18 weeks and median survival calculated from the start of therapy was 28 weeks. Combined chemoimmunotherapy of head and neck cancer with methotrexate, isoniazid, and Glaxo BCG has been reported by Donaldson (24,25). A total of 32 patients with squamous carcinoma of the head and neck had a 62% overall response rate with a median duration of response of 37 weeks calculated from the start of chemotherapy. There was no control group, but compared with other results from the literature these results were impressive. Another lead which could be incorporated into future studies is the increased effect of BCG in the nodes draining the site of administration (16).

Future considerations for the treatment of this tumor would include the use of methotrexate in combination with some of the agents in this protocol in a reduced dosage in such a way that efficacy might be increased and hematologic toxicity decreased. Adjuvant studies should also be tried with similar regimens in high-risk patient groups following primary treatment. An effort to apply BCG or some other nonspecific immunostimulant into the regional nodal drainage would be a logical next step in immunotherapy which should also be considered in an adjuvant setting. A number of other immunologic agents are becoming available (26) and will also deserve trials in the therapy of this very difficult tumor.

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Intracarotid BCNU (NSC-409962): A Toxicity Study in Six Rhesus Monkeys[1,2,3]

David C. Crafts, Victor A. Levin,* and Surl Nielsen[4]

SUMMARY

Intracarotid artery (ica) BCNU was given at doses of 1–20 mg/kg/week for 4 weeks in six rhesus monkeys. Vascular, neurologic, and ophthalmic toxic effects were minor. The monkey receiving a dose of 15 mg/kg had transient left hemiparesis and slowing in her electroencephalogram which cleared 1 week after the third ica infusion of BCNU. At autopsy no vascular, neural, or ophthalmic lesions were found. At doses of 20 mg/kg for two injections, one monkey developed fatty metamorphosis of the liver and Alzheimer II glia in the brain, yet did not develop arteriolitis, corneal damage, or neurologic deficit. It is concluded that reasonable doses of BCNU (less than the systemic maximum tolerated dose) should be well tolerated by ica administration in man.

[Cancer Treat Rep 60:541–545, 1976]

Because of the inadequacy of other forms of treatment, many attempts were made in the 1950s and early 1960s to improve chemotherapy of malignant brain tumors by intracarotid artery (ica) administration (1–4). Mechlorethamine, cyclophosphamide, melphalan, thioTEPA, vincristine, vinblastine, and methotrexate were among the drugs given by this route. Despite optimistic early reports, the benefits were limited and were outweighed by the complications and inconveniences of the indwelling carotid catheters.

Recently, we designed two preclinical studies to approach the possible use of ica-BCNU therapy. First, a pharmacokinetic study was undertaken to determine the relative advantages of ica over iv administration of BCNU and CCNU[5] by following the time course and extent of uptake of ^{14}C isotope into drug- and nucleic acid-associated radioactivity in various regions of squirrel monkey brains. Preliminary results from this study have been reported (5) and a detailed report will follow. So far, our findings indicate that because of the rapidity of blood-brain transport of BCNU and CCNU and the speed of spontaneous chemical transformation to alkylating moieties, ica therapy is potentially four times more effective than iv administration of equal amounts of drug to tumors lying within the territory of a main cerebral artery. Theoretically, this would mean that smaller amounts of drug could be given by the ica route compared to the iv route to obtain the same effect on the tumor.

Our second study, reported here, was an investigation of the toxicity to the carotid artery and brain of BCNU administered on a weekly dose schedule to rhesus monkeys. Ica administration of BCNU in dogs has been evaluated by DeWys and Fowler who found that animals infused with doses of 2–4 mg/kg developed local toxicity consisting of necrotizing arteriolitis, corneal and retinal edema, thinning and mineralization of corneal epithelium, and neurologic

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[3]BCNU: CAS reg. No. 154–93–8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-. Supplied by Drug Research and Development Program, Division of Cancer Treatment, NCI.

[4]Departments of Neurological Surgery (*Drs. Crafts and Levin*), Neurology (*Dr. Levin*), Pharmaceutical Chemistry (*Dr. Levin*), and Pathology (*Dr. Nielsen*), University of California Medical and Pharmacy Schools, San Francisco.

**Reprint requests to*: Victor A. Levin, MD, Brain Tumor Research Center, Department of Neurological Surgery, University of California School of Medicine, San Francisco, Calif 94143.

[5]CCNU: NSC-79037; CAS reg. No. 13010–47–4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-.

deficits (6). Although the doses caused only moderate myelotoxicity, the BCNU may have reached excessively large concentrations in the carotid-artery distribution because of the relatively small canine carotid artery and the restricted anterior cerebral field which it supplies. In contrast, the carotid artery of the monkey and of man supplies a much larger area relative to the size of the animal. Consequently, we decided to administer ica BCNU to six rhesus monkeys at doses of 1–20 mg/kg/week for 4 weeks while following the animals for signs of drug toxicity.

METHODS

Six female rhesus monkeys (*Macaca mulatta*) weighing between 3.8 and 5 kg were to be treated with four injections of a given dose of BCNU at 1-week intervals and killed for histologic examination 3–7 days after the last injection. However, monkey 6 became too ill for further treatment following her second injection and died 2 weeks later. Each week the six monkeys received by ica infusion 1, 1.5, 2, 10, 15, or 20 mg/kg of BCNU respectively.

For each injection the monkey was anesthetized with ketamine and pentobarbital, the neck was prepared with iodine solution, and the right carotid bifurcation was surgically exposed. Immediately prior to the first ica injection for each animal the external carotid was identified and permanently ligated. A 30-gauge lymphangiography needle was inserted into the common carotid artery and BCNU was injected over a 10-minute period. Because of the possibility of infection penicillin and streptomycin were given prophylactically. There were no apparent infections except for superficial cellulitis around the sutures which was minimal when stainless steel sutures were used on the last five animals.

For monkeys 1–4, BCNU was dissolved in absolute ethanol, 1 ml of ethanol/33 mg of BCNU. Saline was added to achieve a 10% ethanol solution. To avoid excessive volume, monkey 5 (15 mg/kg × 4) received 63 mg of BCNU in 1.4 ml of ethanol and 13.5 ml of saline; monkey 6 (20 mg/kg × 2) received 100 mg of BCNU in 1.4 ml of ethanol and 13.5 ml of saline. No solubility problem was encountered. Volumes for injection ranged from 1.5 to 15 ml, depending on the amount of drug given.

Before each injection and immediately before death, blood was drawn for determination of hematocrit levels and white blood cell (wbc), differential, and platelet counts; in addition, blood chemistries were obtained on monkeys 5 (15 mg/kg × 4) and 6 (20 mg/kg × 2).

The animals were observed for any evidence of seizures or neurologic deficit (such as decreased or poor use of the left extremities), for corneal opacity, and for general health as judged by alertness, activity, and food and water consumption. Monkeys 5 and 6 were examined ophthalmoscopically at the time of each treatment. Following death by iv barbiturate, the brain, right eye and optic nerve, and right carotid artery were placed in formalin for histologic examination. The liver, kidneys, adrenals, and lungs of monkey 5 and the liver, kidneys, and infraorbital skin of monkey 6 were also taken.

Monkey 4 was evaluated with right carotid angiography before being killed 7 days after the last treatment. An electroencephalogram (EEG) was performed on monkey 5 just before the third and fourth treatments.

RESULTS

Clinical.—Monkeys 1–4 each received four injections without incident. They showed no change in behavior apart from the transient effects of anesthesia. There was no apparent corneal clouding or visual difficulty and no apparent neurologic deficit except for a right-sided Horner's syndrome related to the surgery. Monkey 5 showed mild listlessness and anorexia for several days after the first injection; by Day 7 this had improved, but erythema was noted in the right periorbital area. After the second injection listlessness and anorexia recurred and were more marked; periorbital redness increased. By Day 13 the skin around the right eye was beginning to desquamate, and a mild tendency to use the right side more than the left was noted, although left extremities could still be used easily. An EEG at this time showed a mild right hemispheral slowing (theta > delta). After the third treatment (Day 14) listlessness and preference for use of the right extremities became more pronounced, although use of the left side improved after Day 16. Prior to the fourth treatment a repeat EEG was normal. Marked weight loss was apparent and oral intake was poor although it improved with fresh-fruit feedings. Lethargy, cachexia, and a mild preference for use of the right upper extremity (although the left upper extremity seemed to function equally as well when it was used) remained through the fourth treatment; by Day 26, 5 days after the fourth treatment, the monkey became stuporous and was killed. Ophthalmic examination revealed no abnormalities at each treatment; corneal clouding was seen only on the last day, when it prevented examination of both eyes, and therefore was probably related to failure of blinking in the preterminal state rather than to BCNU. Monkey 6 tolerated her first two doses of BCNU (20 mg/kg, 100 mg total each dose), but 3 days after the second dose she

TABLE 1.—Dose, myelotoxicity, clinical features, and autopsy findings in 6 monkeys who received ica-BCNU therapy

Monkey No.	Dose/injection		Total dose		Nadir counts (cells/mm³)		Clinical features†	Autopsy findings‡
	Mg/kg	Mg/m²*	Mg	Mg/m²*	Wbc (Day)	Platelet (Day)		
1	1	12	16	46	11,100 (20)	230,000 (21)		
2	1.5	17	28	69	11,300 (1)	294,000 (1)		
3	2	23	37	92	3800 (1)	360,000 (15)		
4	10	115	172	460	3600 (15)	93,000 (28)		Occasional Alzheimer II glia in cerebrum
5	15	172	252	690	1300 (22)	75,000 (22)	Listlessness, anorexia, right periorbital erythema and desquamation, transient left hemiparesis, and focal slowing of EEG during wk 2; killed on Day 26 with BUN > 100 mg/100 ml and LDH 365 IU/liter	Some Alzheimer II glia in cerebrum; some liver regeneration
6	20	230	200	460§	2300 (8)	109,000 (15)	Listlessness, anorexia, and right periorbital erythema and desquamation; no lateralizing signs and death occurred on Day 21	Some Alzheimer II glia in cerebrum; early fatty degeneration of liver

*Computation based on ref 9.
†All animals had Horner's syndrome after surgery.
‡All animals had adventitial fibrosis around carotid artery at injection site.
§Received only 2 treatments.

became lethargic and took food and water poorly. This condition gradually worsened and the third treatment was omitted. Beginning on Day 10 there was erythema and then desquamation of the skin of the right eyelids and infraorbital area. Despite marked lethargy and generalized weakness, no focal or lateralized neurologic deficit could be found. Deep tendon reflexes were brisk but symmetrical, tone was normal, and response to noxious stimuli was grossly intact bilaterally. There was no corneal clouding except at the time of death when it was bilateral. Monkey 6 died 21 days after the first treatment.

Even during light anesthesia, ica injection of BCNU caused painful arousal comparable to that caused by suturing.

Blood counts and laboratory results.—Monkeys 1–3 had no fall in platelet or wbc counts during the treatments. The only low wbc count was noted in monkey 3 before therapy. The wbc count was 3800 cells/mm³ before treatment but rose to > 6000 cells/mm³ in succeeding weeks. In monkey 4 a mild myelosuppression was apparent while monkeys 5 and 6 had a fairly severe depression of blood counts (table 1). Since myelotoxicity from BCNU is delayed it is probable that the blood counts would have fallen further if the experiment had been sustained over a longer period of time. Monkey 5 started with an alkaline phosphatase level of 209 international units (IU)/liter and a lactic dehydrogenase (LDH) level of 192 IU/liter but normal serum glutamic oxaloacetic transaminase (SGOT), bilirubin, and creatinine levels. On Day 15 the LDH level rose to 304 IU/liter and the SGOT level rose to 40 IU/liter, and on Day 22 the alkaline phosphatase level rose to 368 IU/liter. The blood urea nitrogen (BUN) and the enzyme levels rose higher terminally. On Day 15 the creatinine level of monkey 6 was 3.3 mg/100 ml but the bilirubin, SGOT, and alkaline phosphatase levels were normal.

Arteriography.—The right carotid angiogram of monkey 4 on Day 28 is shown in figure 1. There was absence of filling of the right external carotid, as expected, and patency of the internal carotid system. There was a question of narrowing in the petrous portion but this was not confirmed pathologically. No vessel beading was observed.

Pathology.—The brains and eyes of all the animals appeared grossly normal judging by the appearance of both the surface and thin sections. In all cases, multiple sequential sections of the right carotid artery were examined histologically. Microscopic examination of the carotids revealed marked adventitial fibrosis with foreign-body reaction near the site of injection. In several specimens at the site of injection, organized thrombus was present in the lumen but patency of the vessel was maintained. All other levels of the carotid showed maintenance of the integrity of the wall. No active destruction, inflammation, or fibrosis was seen. The ophthalmic, middle and anterior cerebral, and lenticulostriate arteries appeared normal microscopically as did the

FIGURE 1.—*Monkey 4*—Right carotid angiogram on Day 28, 7 days after the last of 4 ica infusions of BCNU. Note absence of right external carotid artery, apparent narrowing of intrapetrous internal carotid artery (no lesion found on dissection), and absence of other narrowings, occlusions, or beading. Retrograde filling of basilar artery probably related to force of injection which was greater than for BCNU.

vessels of the base of the brain and the intrinsic vessels of the brain. The retina, optic nerve, cornea, and ciliary body of each monkey were all without detectable macroscopic lesions. From the optic nerve of monkey 5 sections were made at 1-mm intervals and no abnormalities were discovered. Multiple areas of cerebral white and gray matter and basal ganglia showed no evidence of emboli, intrinsic vessel disease, or infarction of cerebral parenchyma. The cortical ribbon was uniform throughout both hemispheres. In monkey 4 and especially in monkey 6 an increased number of Alzheimer II glia were seen. The Alzheimer II glia are commonly seen in metabolic encephalopathies (particularly hepatic). The liver of monkey 6 showed normal lobular architecture without inflammation or cholestasis. Mild nuclear pleomorphism and double cell plates suggested regeneration of hepatocytes. All hepatocytes showed microvacuolation or macrovacuolation which is typical of fatty metamorphosis. The liver of monkey 5 showed normal architecture and no necrosis but considerable nuclear pleomorphism of individual hepatocytes. Many liver-cell cords were two or four cell plates suggesting regeneration. The kidneys of monkey 6 appeared normal histologically, as did the kidneys, lungs, and adrenals of monkey 5. The skin from the infraorbital area of monkey 6 showed thinning of the epidermis. Squamous cells in the basal, malpighian, and granular layers had large vesicular irregular nuclei with prominent nucleoli.

DISCUSSION

BCNU given iv has been an effective agent in the therapy of malignant brain tumors (7,8). Its rapid tissue fixation and lipid solubility make its intra-arterial use attractive since a lower dose with less myelosuppression could, theoretically, give the same tumor dose as the standard iv treatment (5). Ica BCNU in dogs caused neurologic deficits (8–15 days after treatment) and necrotizing arteriolitis of the internal carotid system (6). The doses were 2–4 mg/kg/dose in dogs weighing 19.8–38.2 kg. These doses,

whether administered iv or ica, were large enough to cause significant myelosuppression and were probably equivalent to an iv dose of at least 200 mg/m² in man (9). Intra-arterial administration did not reduce myelotoxicity compared to iv administration. Notably, ica ethanol without BCNU, equal in amount to that given with BCNU, did not cause local toxicity.

In our study four injections per animal at 1-week intervals were given. We were not interested in analyzing the time course of myelosuppression but rather in observing any vascular and neurologic toxicity which might develop 3–4 weeks after ica BCNU. We did not want to miss changes such as arteriolitis which potentially could occur and resolve within days to several weeks after an injection. In addition, we wanted to note the effects, if any, of repetitive doses.

When no unfavorable results were seen at doses of 1–2 mg/kg intra-arterially × 4, the dose was markedly increased in order to reproduce the local toxicity seen by DeWys and Fowler (6) in dogs. At doses of 10 mg/kg × 4, however, there was still no evidence of local side effects. At doses of 15 mg/kg × 4 a transient hemiparesis was observed, that was confirmed by the right-sided EEG slowing after two treatments. This hemiparesis cleared, along with the EEG abnormality, by the fourth treatment and it is unclear whether it was related to the BCNU or to a technical problem in the injection (embolus, ischemia). No pathologic explanation was found grossly or histologically. At a dose of 20 mg/kg (100 mg), a dose which fairly rapidly produced systemic toxicity with liver and kidney damage (common toxic effects of nitrosoureas in monkeys although rarely significant in man),[6] followed by lethargy and death, no corneal opacity or focal neurologic disorder was seen. However, skin desquamation in the right infraorbital area (supplied by the internal carotid artery) was observed. Thus, the limiting toxicity causing death or a moribund state in monkeys 5 and 6 was systemic (related to the total dose) rather than focal (related to the route of administration). Of interest is the fact that a single dose of 20 mg/kg in the monkey would be equivalent to 230 mg/m² in man (9). In our study, this dose was given twice in 1 month adding up to a far higher total dose than would be given to man because of associated myelotoxicity. The cumulative monthly dose was 460 mg/m² in monkeys 4 and 6 and 690 mg/m² in monkey 5, which would be equivalent to two and three times the usual human monthly dose respectively.

[6]Broder LE, and Carter SK. Clinical brochure: 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) NSC-409962. Prepared by the NCI, Bethesda, Md, Sept 1970, 84 pp.

There are several possible explanations for the discrepancies between the results with dogs and those from the present study involving monkeys. A difference in the drug is conceivable, but the BCNU for this investigation was supplied by the NCI; samples from the same lot were shown to be > 99% chemically pure by high-pressure liquid chromatography and were as effective as expected in the treatment of tumor-bearing rodents.

While it is possible that there is a species (dog vs monkey) difference in tissue susceptibility, it is likely that the local tissue concentration of drug on the initial passage through the brain is responsible for differences in observed dose-dependent toxicity in the dog. The dose was injected into the internal carotid artery in both studies and was calculated according to body weight. In the dog, the internal carotid has a very limited distribution. In the monkey and in man the internal carotid has a much wider cerebral hemispheral distribution so that each arteriole would receive a smaller proportion of the entire injected dose than would be the case in the dog.

Extrapolation of our animal studies to man would suggest that ica BCNU doses of 115 mg/m² or less should be well tolerated. This would be most useful for those patients whose tumor is entirely within the distribution of one carotid artery and in whom myelotoxicity is a limiting factor.

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Experimental Chemotherapy With 5-Arylpyrimidine Antifolates: Preliminary Studies of Toxicity and Responsiveness of Sarcoma 180 to DDMP (NSC-19494) and DDMP With Citrovorum Factor (NSC-3590)[1,2,3]

F. M. Sirotnak,* D. M. Dorick, and D. M. Moccio[4]

SUMMARY

The 5-arylpyrimidine antifolate DDMP showed appreciable therapeutic activity against an ascitic form of Sarcoma 180 in $BD2F_1$ mice. Antitumor effects were highly schedule and dose dependent at a limited number of doses within the range of 8–40 mg/kg. The best results (increased lifespan = 112%) were obtained with two doses of DDMP at 40 mg/kg given 4 days apart. The incorporation of citrovorum factor rescue in dose schedules with DDMP appeared to improve the therapeutic index. In multiple-dose schedules with citrovorum factor allowing an average of eight doses of DDMP at a maximum level of 16 mg/kg, increases in median lifespan were > 158% with a number of long-term survivors.

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Methotrexate and related folate analogs are highly effective in treating some experimental neoplasms and certain forms of human cancer (1–7). The level of effectiveness observed in these cases undoubtedly bears some relationship to the unique multifactorial role (8) of the target enzyme dihydrofolate reductase in macromolecular synthesis. Recent evidence (9,10) from our laboratory confirms earlier work (11–13) incriminating the membrane-transport mechanism for antifolate permeation as a major determinant of relative responsiveness among murine tumors. Other evidence (9,14) from our laboratory associates this property with the selectivity of action of this class of agent as well.

In the case of tumors unresponsive to methotrexate by virtue of a deficiency in membrane transport, an antifolate drug which because of high lipophilicity gains entry by passive diffusion may be a useful alternative. Also, such an agent could conceivably provide additional therapeutic advantages when used in conjunction with citrovorum factor rescue, a procedure successfully used to increase the therapeutic index of methotrexate (1,2). Since the natural folate, as with methotrexate, also requires carrier-mediation for entry (15), therapy with a lipophilic antifolate plus citrovorum factor would involve a different basis for penetration of each entity. Preliminary indication of the usefulness of this approach has been derived during therapy of Sarcoma 180 with the 5-arylpyrimidine DDMP. Data related to some aspects of this work have been given in an earlier report (16).

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[3]DDMP: CAS reg. No. 7761-45-7; pyrimidine, 2,4-diamino-5-(3,4-dichlorophenyl)-6-methyl-. Supplied by Wellcome Research Laboratories, Research Triangle Park, NC.

Citrovorum factor: CAS reg. No. 6035-86-5; glutamic acid, *N*-[*p*-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl]-, calcium salt; leucovorin.

[4]Laboratory for Molecular Therapeutics, Memorial Sloan-Kettering Cancer Center, New York, NY.

**Reprint requests to*: F. M. Sirotnak, PhD, Laboratory for Molecular Therapeutics, Sloan-Kettering Institute for Cancer Research, 145 Boston Post Rd, Rye, NY 10580.

MATERIALS AND METHODS

Ascitic forms of the Sarcoma 180 line (obtained from Dr. G. Chihara, National Cancer Research Institute, Tokyo, Japan) were maintained (7) by transplantation ip in female $BD2F_1$ mice (C57BL female/DBA_2 male, obtained from A. R. Schmidt, Madison, Wis). For experiments, new lines were initiated in $BD2F_1$ mice from a bank of frozen cells prepared earlier from a line determined to be malignant and maintained for only five transplantation generations and then discarded.

TABLE 1.—Multiple-dose toxicity of DDMP in BD2F, female mice

DDMP* dose (mg/kg)	No. of experiments	No. of toxic deaths/total No. of mice						
		Dosage interval (days)						
		1	2	3	4	5	6	7
2	2	0/10	0/10	0/10	0/10	0/10	0/10	0/10
4	2	0/10	0/10	0/10	0/10	0/10	0/10	0/10
8	3	10/10	15/15	13/20	4/20	0/15	0/10	0/10
16	3	10/10	20/20	20/20	10/20	3/15	0/10	0/10
24	3	10/10	10/10	15/15	15/15	9/15	0/10	0/10
32	2	10/10	10/10	10/10	10/10	8/10	6/10	0/10
40	2	10/10	10/10	10/10	10/10	10/10	8/10	0/10

*DDMP given for a total of 5 doses.

DDMP was administered sc in the scapular region as the HCl salt in aqueous solution (pH 5.1). Citrovorum factor was also administered sc at the same site as the calcium salt in neutral aqueous solution. During therapy experiments, mice (20 g) were inoculated ip with 10^6 cells (0.1 ml) and randomly distributed among control and treated groups. Animals were maintained on Purina laboratory chow and water ad libitum. Untreated animals with ascites Sarcoma 180 died within 10–13 days. During drug toxicity determinations animals were observed for a total of 21 days. All experiments were repeated two to eight times.

RESULTS

Single-Dose Toxicity of DDMP

Groups of five mice each were administered single doses of DDMP in amounts varying from 10 to 100 mg/kg. As expected for a highly lipophilic agent, lethality appeared to be related to neurologic toxicity. At a dose > 40 mg/kg all animals had convulsions within 15 minutes of injection. Toxic deaths were dose related between 45 and 70 mg/kg with death occurring usually within 3 hours of injection. The LD50 was 50–55 mg/kg. A preliminary study on single-dose toxicity of DDMP in animals has been reported elsewhere (17).

Multiple-Dose Toxicity of DDMP

Data were derived following the administration of DDMP at several dose levels given at varying time intervals. The results of an experiment in which mice were given a total of five injections are shown in table 1. At doses of 4 mg/kg or less no toxicity was observed when DDMP was given as often as once per day. At higher doses toxic deaths were observed when the drug was given at intervals of 4 days or less (8 mg/kg), 5 days or less (16 and 24 mg/kg), or 6 days or less (32 and 40 mg/kg). On regimens in which toxic deaths occurred, weight loss was not appreciable within the first 7 days (10% or less), but at the time of death 3–7 days later the average weight loss was 30%–40%. As in the case of other antifolates (18) multiple-dose toxicity from DDMP appears to be associated with effects at host-renewal sites in the small intestine. On gross pathologic examination after death this organ had a fragile paper-thin appearance and weighed half that of the normal organ. Preliminary indications of drug-related degeneration in the small intestine and to a lesser extent in the bone marrow have been reported earlier (17).

Responsiveness of Sarcoma 180 to DDMP Alone

No therapeutic effect against Sarcoma 180 was observed at any dose level using schedules in which DDMP could be given for an unlimited number of doses without toxicity. However, a therapeutic response was obtained when DDMP was given at a normally toxic level but in a limited number of doses. Effective regimens were sought, therefore, in experiments in which dose levels were maximized by optimization of dosage interval. A summary of the results obtained when therapy was limited to only two doses of varying amounts of DDMP is shown in figure 1. Antitumor effects generally occurred between the range of 8–40 mg/kg. However, as the dose was increased a longer interval was required to avoid toxicity and to obtain the greatest increase in survival time (112% with 40 mg/kg given 4 days apart).

The relationship between the dose level and the number of doses of DDMP was also determined for intervals of 2–5 days. At intervals of 2 days or less more than two doses of DDMP at 8 mg/kg or above were toxic. Doses of 4 mg/kg or less given repeatedly at intervals as small as 1 day gave no therapeutic effect. For these reasons, and in view of the fact that maximum therapeutic effects at the highest tolerated dose were observed at longer intervals (up

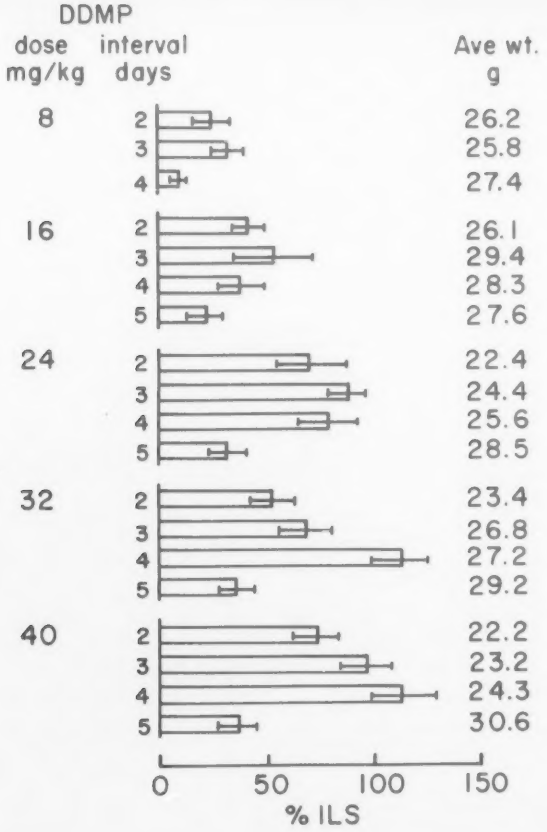

FIGURE 1.—Schedule dependence of response to therapy with DDMP at different doses. Data shown are averages based on 2–4 separate determinations in which groups of 5 mice each were given 2 doses of DDMP sc at the levels shown at 2-, 3-, 4-, or 5-day intervals. The average weight for each group at the time of death is also shown (initial weight = 20 g). Survival time is expressed as percentage ILS ± SD.

to 4 days) (fig 1), only the results obtained at 3 or 4 days are shown (tables 2 and 3). At a 3-day interval mice tolerated three doses of DDMP at 8 mg/kg with a > 50% increase in lifespan (ILS) (table 2). Four doses at this same level were toxic. The antitumor effects observed after two doses of 16 or 24 mg/kg were about twice that obtained with one dose at the same level. Three or four doses at each level were toxic. Three doses at 24 mg/kg were still not tolerated at 4-day intervals (table 3). Antitumor effects of two doses of 32 mg/kg were about twice that obtained with one dose, but three doses were toxic. At the same interval between doses two doses of 40 mg/kg gave the greatest ILS. Three doses at this level were toxic.

Multiple-Dose Toxicity of DDMP With Citrovorum Factor Rescue

The effect of citrovorum factor administration at different doses on toxicity due to lethal doses of DDMP during multiple-dose therapy is shown in table 4. DDMP was given at a dose of 16 mg/kg once every 2 days for a total of 5 days. Some reduction in toxic deaths was observed with citrovorum factor doses of 6–24 mg/kg given simultaneously with DDMP or 8 hours later. Further reduction was observed at the same citrovorum factor doses given 16, 24, or 32 hours later. As seen in tables 4 and 5 the higher the citrovorum factor dose and the greater its frequency of administration following DDMP, the fewer the toxic deaths. More citrovorum factor was required to prevent toxic deaths when the DDMP dose was increased.

Responsiveness of Sarcoma 180 to DDMP With Citrovorum Factor Rescue in a Preliminary Trial

Dose levels of 8, 16, 32, and 40 mg/kg of DDMP were selected for trials with citrovorum factor with the objective of achieving an increase in the total number of doses tolerated with smaller intervals. Based on the data for multiple-dose toxicity (tables 1, 4, and 5) multiple-dose schedules were tested in which the drug was given at either 2- or 3-day intervals accompanied by either a single dose of citrovorum factor given 24 hours after each dose of DDMP (3-day intervals) or two doses of citrovorum factor given 24 and 32 hours after each dose of DDMP (2-day intervals). The data are shown in table 6. Schedules using simultaneous administration of DDMP and citrovorum factor were not used since the data shown in table 4 suggested this was less effective in preventing toxicity. Each treatment schedule was also evaluated with regard to citrovorum factor dose. It is important to note that DDMP was administered only on an elective basis, that is, drug was administered only if weight loss did not exceed 15%. In the event of further weight loss, DDMP administration was discontinued until the weight returned to 17 g. However, during the interim period the specified regimen of citrovorum factor was continued. The results are shown in table 6.

The most effective schedules used DDMP at a dose of 16 mg/kg given every 3 days (followed by one dose of citrovorum factor). The greatest ILS was obtained when this schedule was used with citrovorum factor concentrations of 6 or 12 mg/kg. In addition, a number of long-term survivors were also obtained. Both schedules using DDMP every 2 days (8 or 16 mg/kg DDMP with citrovorum factor) gave

TABLE 2.—Response of ascites Sarcoma 180 to DDMP given on an every-3-day schedule

No. of mice*	DDMP dose (mg/kg)	No. of doses†	MST ± SD (days)	ILS (%)	Average weight in g at death‡ (approximate range)
10 × 4	—	—	11.2 ± 1.3	—	26.5 (24–29)
5 × 2	8	1	12.2 ± 0.7	10	26.4 (22–30)
5 × 3		2	14.4 ± 1.6	34	25.7 (21–31)
5 × 3		3	16.9 ± 1.3	52	23.7 (15–30)
5 × 3		4	12.1 ± 1.4	9	17.4 (13–26)
5 × 2	16	1	13.7 ± 1.9	21	26.5 (19–32)
5 × 4		2	16.7 ± 1.8	49	24.2 (17–30)
5 × 4		3	16.1 ± 1.4	43	19.2 (14–29)
5 × 2		4	12.1 ± 0.6	8	15.4 (12–17)
5 × 2	24	1	14.9 ± 1.1	34	27.1 (23–31)
5 × 3		2	20.1 ± 1.8	79	26.1 (17–35)
5 × 2		3	15.4 ± 1.7	39	19.6 (17–28)
5 × 2		4	11.8 ± 0.8	5	15.1 (14–17)

*No. of mice in each experiment × No. of experiments.
†Given sc as a single injection.
‡Initial weight = 20 g.

TABLE 3.—Response of ascites Sarcoma 180 to DDMP given on an every-4-day schedule

No. of mice*	DDMP dose (mg/kg)	No. of doses†	MST ± SD (days)	ILS (%)	Average weight in g at death‡ (approximate range)
10 × 3	—	—	11.4 ± 0.7	—	27.6 (23–32)
5 × 3	24	1	19.8 ± 1.6	30	26.2 (22–31)
5 × 3		2	18.7 ± 1.3	64	25.9 (23–30)
5 × 2		3	18.0 ± 1.7	58	20.3 (16–28)
5 × 3	32	1	16.1 ± 1.2	41	28.4 (26–32)
5 × 4		2	22.9 ± 2.4	103	31.2 (25–37)
5 × 2		3	11.8 ± 2.6	4	18.5 (14–24)
5 × 3	40	1	16.4 ± 2.1	44	27.3 (23–30)
5 × 6		2	24.2 ± 2.7	112	24.2 (14–33)
5 × 2		3	12.6 ± 1.1	11	16.4 (14–20)

*No. of mice in each experiment × No. of experiments.
†Given sc as a single injection.
‡Initial weight = 20 g.

the best results at citrovorum factor concentrations of 3–12 mg/kg. The average ILSs in these cases were lower and there were fewer long-term survivors. The schedules using DDMP at a dose of 32 mg/kg every 3 days with citrovorum factor given 24 hours later were generally unsatisfactory because of toxicity. The same result was obtained with DDMP at doses of 32 and 40 mg/kg every 4 days with citrovorum factor given 24 hours later. These data are not shown.

DISCUSSION

A therapeutic response of the ascites Sarcoma 180 to DDMP could be shown when DDMP was used alone or in combination with citrovorum factor. The maximum responsiveness was highly schedule dependent and required a very specific adjustment of dose level with respect to dose interval for DDMP. This was obtained with dose intervals which permitted therapy at the highest sublethal level (40 mg/kg)

TABLE 4.—Effect of citrovorum factor administration on the toxicity of DDMP (16 mg/kg) given in multiple doses

Interval* (hrs)	No. of toxic deaths/total No. of mice†					
	Citrovorum factor (mg/kg)					
	—	1.5	3.0	6.0	12.0	24.0
0	20/20	10/10	10/10	8/10	10/10	6/10
8	—	10/10	10/10	8/10	8/10	5/10
16	—	10/10	10/10	7/10	5/10	3/10
24	—	10/10	8/15	8/15	6/15	2/15
32	—	10/10	10/10	8/10	7/10	4/10

*Citrovorum factor given sc with DDMP (16 mg/kg sc q2days for 5 injections) or 8, 16, 24, or 32 hrs after each dose.
†Two experiments.

TABLE 5.—Effect of citrovorum factor administration on the toxicity of different doses of DDMP

Schedule	DDMP dose (mg/kg)	No. of toxic deaths/total No. of mice*						
		Citrovorum factor (mg/kg)						
		—	1.5	3.0	6.0	12.0	24.0	48.0
1†	8	20/20	10/15	8/15	2/15	0/15	0/15	—
	16	20/20	10/10	14/20	11/20	8/20	0/15	—
	32	20/20	10/10	11/15	10/15	10/15	10/15	5/10
2‡	8	20/20	2/15	0/15	0/15	0/15	0/15	—
	16	20/20	6/20	6/20	0/15	0/15	0/15	—
	32	20/20	11/15	11/15	11/15	10/15	10/15	2/10

*Two experiments.
†One dose of DDMP sc q2days for a total of 5 doses followed by 1 dose of citrovorum factor 24 hrs later.
‡One dose of citrovorum factor given with DDMP and 8, 24, and 36 hrs later.

in a limited number of doses (usually two). Multiple-dose regimens with citrovorum factor permitted a greater number of doses of DDMP but could only be optimized at a DDMP level (16 mg/kg) substantially below the lethal dose. It should be emphasized that during the current studies no attempt was made to optimize schedules with respect to a large number of variables; we were attempting to provide preliminary documentation for the marked schedule dependence of this form of therapy and the potential for improvement in therapeutic index with citrovorum factor rescue. The data related to the use of DDMP alone confirm earlier indications of the antitumor properties of this class of drug reported some years ago and more recently (19–21).

An apparent improvement in the therapeutic effect of DDMP with citrovorum factor is only suggested by the data. However, a somewhat better overall increase in median survival time (MST) was obtained when compared to other regimens in which DDMP could be given at higher doses alone. Also, long-term survivors (45-day survivors in table 6) were obtained with DDMP plus citrovorum factor on a multiple-dose schedule and, for this reason alone, such a schedule might be a preferable alternative. As a consequence of the neurologically related single-dose toxicity, there is an upper limit of the DDMP dose which can be used with citrovorum factor. Therefore, this modality cannot be applied in exactly the same way as it is with methotrexate (1,2) where citrovorum factor administration allows a single dose in the lethal range. The two remaining options examined here, namely, a shorter dose interval and an increase in the total number of doses of citrovorum factor, will require more elaborate testing. The basis of the protection against multiple-dose toxicity of DDMP by citrovorum factor will also require further study for elucidation.

The data provided here are of some significance in view of recent clinical reports (22–24) suggesting the possible therapeutic utility of the 5-arylpyrimidine antifolates against human cancer. This is in contrast to very early clinical studies (25) in which the clinical effectiveness of DDMP could not be shown. It is hoped that the laboratory data provided with this murine sarcoma model might stimulate further laboratory and clinical interest particularly with regard to trials with DDMP plus citrovorum factor.

TABLE 6.—Response of ascites Sarcoma 180 to DDMP with citrovorum factor rescue

Schedule*	DDMP dose (mg/kg)	DDMP dose interval (days)	No. of doses†	Citrovorum factor dose (mg/kg)	MST ± SD‡ (days)	ILS (%)	Average weight in g at death§ (approximate range)	45-day survivors¶
Control	—	—	—	—	11.2 ± 0.8	—	26.2(23–31)	0/40
1	8	2	3–4	—	10.9 ± 1.0	−3	15.2(14–17)	0/10
			3–4	1.5	11.2 ± 1.6	0	21.0(17–28)	0/10
			4–5	3.0	13.0 ± 1.2	16	18.5(16–21)	0/10
			4–10	6.0	>24.4 ± 2.6	>117	23.2(18–29)	1/10
			4–9	12.0	>23.2 ± 1.7	>107	22.5(18–23)	1/10
			4–5	24.0	11.3 ± 0.9	0	24.2(21–26)	0/10
2	16	2	3–4	—	10.7 ± 1.2	−4	14.8(14–16)	0/10
			3–4	1.5	13.6 ± 1.4	21	16.8(15–22)	0/10
			3–8	3.0	>21.3 ± 1.9	>90	15.0(13–18)	1/10
			3–11	6.0	>24.6 ± 2.6	>118	18.3(16–23)	1/10
			4–5	12.0	14.0 ± 1.3	25	20.2(19–21)	0/10
			4–5	24.0	11.9 ± 1.8	6	22.2(18–24)	0/10
3	16	3	3–4	—	14.5 ± 1.9	21	15.8(14–20)	0/10
			3–5	1.5	12.5 ± 2.1	12	17.0(14–20)	0/10
			3–6	3.0	15.1 ± 1.3	35	15.3(13–17)	0/15
			3–12	6.0	>29.1 ± 3.1	>158	17.2(14–21)	3/15
			4–9	12.0	>25.4 ± 2.6	>126	20.8(17–25)	3/15
			4–8	24.0	12.1 ± 0.6	8	22.4(18–24)	0/10
4	32	3	3–4	—	13.4 ± 0.9	11	15.6(14–19)	0/10
			3–4	1.5	10.3 ± 1.2	−8	16.2(14–18)	0/10
			3–4	3.0	14.5 ± 1.6	30	20.6(14–23)	0/15
			3–4	6.0	>19.0 ± 2.3	>69	19.5(15–25)	1/15
			3–4	12.0	11.2 ± 0.7	0	19.2(16–21)	0/10
			3–4	24.0	14.2 ± 1.7	29	20.7(17–22)	0/10

*Citrovorum factor given sc 24 hrs (schedules 1, 3, 4) or 24 and 32 hrs (schedule 2) after each sc dose of DDMP.
†DDMP given until weight loss = 15%.
‡Two to eight groups of 5 mice each, not including 45-day survivors.
§Initial weight = 20 g.
¶Nonascitic on the 45th day.

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Pharmacologic Disposition of Inosine Dialdehyde (NSC-118994) in Mice, Rats, Dogs, and Monkeys[1,2,3]

Richard L. Cysyk* and Richard H. Adamson[4,5]

SUMMARY

The pharmacologic disposition of inosine dialdehyde (IdA) was studied in mice, rats, dogs, and monkeys. ^{14}C-IdA was utilized to follow plasma and tissue levels and patterns of excretion. Plasma radioactivity curves were multiphasic with an initial rapid component (half-life = 12–18 mins) followed by a much slower decline (half-life = approximately 3 days). As time progressed after the injection, an increasing fraction of the total plasma radioactivity was found to be associated with plasma proteins and was trichloroacetic acid (TCA) insoluble. IdA interacts extensively with proteins and small molecules both in vivo and in vitro, forming complexes that do not dissociate under physiologic conditions. The pharmacologic disposition of IdA is dependent upon the relative extent of IdA interaction with protein macromolecules versus small transportable molecules. The major route of excretion was urinary with > 50% of the dose excreted during the first 24 hours after iv administration in all species studied. IdA was recovered in the urine as complexes with urine constituents. There was no preferential uptake of IdA by any organ. All radioactivity remaining 24 hours after IdA administration was associated with plasma and cellular proteins and was nondialyzable and TCA insoluble. IdA was not a substrate for aldehyde oxidase and did not inhibit the activity of this enzyme.

[Cancer Treat Rep 60:555–562, 1976]

Periodate oxidation of ribonucleosides results in the formation of dialdehydes (1) which have demonstrated cytotoxic properties (2). Inosine dialdehyde (IdA; fig 1), the periodate-oxidation product of inosine, is highly active against several transplantable murine tumors including L1210[6] and P388 leukemias and several variants of these tumors resistant to commonly used cancer chemotherapeutic agents.[7] IdA is presently undergoing phase I clinical evaluation at cancer centers (3). A study of the pharmacology of IdA in several species was undertaken in our laboratory to aid in this evaluation. The results of the pharmacologic disposition studies of IdA in mice, rats, dogs, and monkeys are presented in this report and a study of the interaction of IdA with proteins and low molecular weight compounds is presented in the following paper (4).

MATERIALS AND METHODS

^{14}C-IdA (2.74 millicuries/mmol, labeled in the 8 carbon of the hypoxanthine moiety) (fig 1), synthesized by Stanford Research Institute, and IdA were obtained from the Drug Research and Development Program, DCT, NCI. NCS tissue solubilizer used in these studies was purchased from Amersham/Searle Corp, Arlington Heights, Ill, and toluene-^{14}C (4.17 ×

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[2]A preliminary report of this work has appeared as an abstract: Cysyk RL, and Adamson RH. Anti-tumor properties and pharmacologic disposition of inosine dialdehyde. Proc Am Assoc Cancer Res and ASCO 15:56, 1974.

[3]Inosine dialdehyde: diglycolaldehyde, α-(hydroxymethyl)-α′-(6-hydroxy-9*H*-purin-9-yl)-.

[4]Laboratory of Chemical Pharmacology (LCHPH), Experimental Therapeutics Program (ETP), Division of Cancer Treatment (DCT), National Cancer Institute (NCI), Bethesda, Md.

[5]The technical assistance of Miss Mary Ellen Ensminger and Mrs. Ovella Ayers is appreciated. We wish to thank Dr. David G. Johns for performing the aldehyde oxidase studies.

[6]Screening data from Drug Research and Development Program, DCT, NCI.

**Reprint requests to*: Dr. Richard L. Cysyk, LCHPH, ETP, DCT, NCI, Bldg 10, Rm 6N119, National Institutes of Health, Bethesda, Md 20014.

[7]Cysyk RL, and Adamson RH. Antitumor properties of inosine dialdehyde. In preparation.

10^5 dpm/ml) was purchased from New England Nuclear Corp, Boston, Mass. Scintillation solution used for radioactive determinations contained 200 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene, 6 g of 2,5-diphenyloxazole (New England Nuclear Corp), 1400 ml of toluene, and 600 ml of methanol.

Thin-Layer Chromatography

The chemical and radiochemical purity of IdA and ^{14}C-IdA were found to exceed 99% by thin-layer silica gel chromatography (Eastman Chromagram) in systems containing ethyl acetate:ethanol:water (10:10:3) or *n*-butanol:acetic acid:water (4:1:1). It was found that complexes of IdA and urine constituents separated well on thin-layer silica gel plates prewashed and developed in ethyl acetate:ethanol:water (10:10:3). In this system IdA has an R_f value of 0.62. ^{14}C-IdA and complexes containing ^{14}C-IdA were detected by autoradiography using X-ray film and were quantitated by removing the spots and determining the amount of radioactivity.

Studies With Rats and Mice

Plasma and bile levels of radioactivity were determined in male Sprague-Dawley rats (250–300 g). Rats were anesthetized with pentobarbital sodium (45 mg/kg ip), the common bile duct was cannulated with PE-10 tubing, and the abdominal aorta was cannulated above the bifurcation of the common iliacs with a PE-50 cannula held in place with adhesive (5). Rats were sutured with cannulas in place, transferred to restraining cages (6), allowed to recover from anesthesia, and provided with free access to water. Prior to full recovery from anesthesia, ^{14}C-IdA (5–10 microcuries/rat), diluted with nonradioactive IdA in water to yield the final dose in a total volume of 1 ml, was injected into the femoral vein over a 1-minute period. Blood samples were taken into heparinized tubes at the indicated time intervals and 10 μl of plasma was removed for determining total content of radioactivity. Immediately after sampling, the remaining plasma was diluted with an equal volume of cold 20% trichloroacetic acid (TCA) and the radioactivity of the TCA-soluble fraction determined. Bile was collected continuously and sampled periodically for determination of radioactivity.

Male CDF_1 mice (25 ± 1 g; National Institutes of Health breeding colonies) were injected (ip, sc, iv, or orally) with ^{14}C-IdA (1–5 microcuries/mouse). Blood samples were taken by pressing a heparinized Pasteur pipette against the orbital sinus. Radioactivity in the tissues was determined by excising, blotting, and weighing the organs and the entire organ was

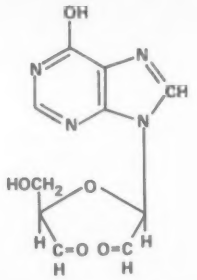

FIGURE 1.—Structure of IdA.

dissolved in a minimum amount of NCS tissue solubilizer at 49°C. Samples (25–50 μl) of the digest were added to 18 ml of scintillation solution and counted in a Beckman LS-230 liquid scintillation counter. Quenching was corrected for by the method of internal standardization using ^{14}C-toluene (7). Rats and mice used in chronic studies were housed in glass metabolic cages with access to food and water. Urine was collected continuously and feces were collected in containers placed over the anal area to prevent their contamination with urine. Urine samples (25–100 μl) were counted directly in scintillation fluid while the feces were homogenized and digested in NCS solubilizer prior to counting.

Studies With Dogs and Monkeys

Female beagle dogs (7.3–8.1 kg) and female rhesus monkeys (4.9–5.9 kg) were anesthetized iv with sodium pentobarbital and light anesthesia was maintained during the first 8 hours of the experiment by periodic injections of pentobarbital. ^{14}C-IdA (about 40 microcuries) was injected into the femoral vein at the dose indicated in a volume of 2 ml over a 1-minute period. Heparin-treated blood samples (2 ml) were collected from a cannulated femoral artery and treated as described above for rats and mice. Urine was collected continuously via an indwelling Foley catheter. Spinal fluid was collected via a 20-gauge needle inserted into the cisterna magna. After 8 hours, the catheter and cannulas were removed and the animals placed in cages for collection of urine and feces for the next 16 hours. Dogs used in the chronic studies were given free access to food and water.

Aldehyde Oxidase Studies

Rabbit liver aldehyde oxidase was purified according to the method of Rajagopalan et al (8) and assayed by the method of Johns et al (9). The assay mixture contained 3',5'-dichloromethotrexate (DCM) (5×10^{-5} M), sodium phosphate buffer (0.03 M, pH

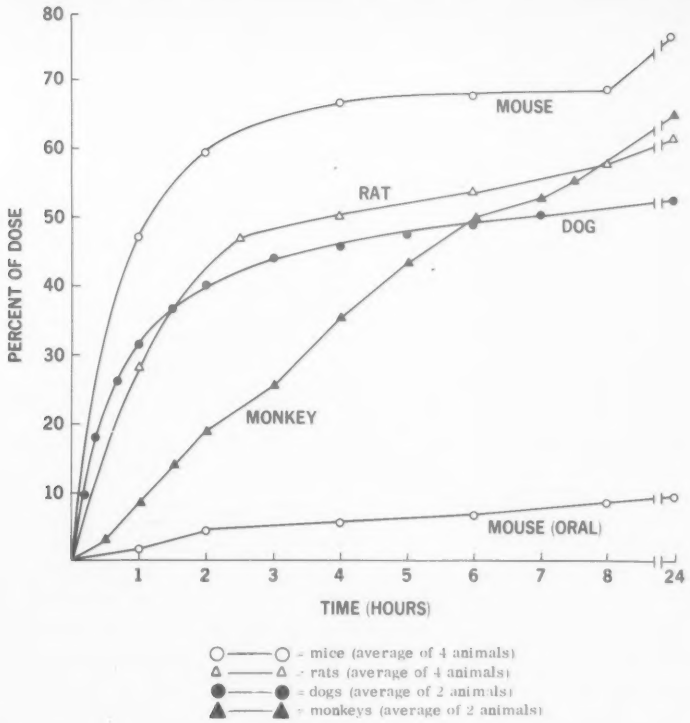

FIGURE 2.—Excretion of radioactivity in the urine following administration of ^{14}C-IdA to dogs (10 mg/kg, iv), monkeys (10 mg/kg, iv), rats (40 mg/kg, ip), and mice (180 mg/kg, ip and orally). Samples were assayed for radioactivity as described in the Materials and Methods section and expressed as percent of dose administered.

7.5), and enzyme. The increase in absorbance at 340 nm that results from the formation of 7-hydroxy-DCM was followed with a Guilford recording spectrophotometer. IdA, at concentrations ranging from 1×10^{-5} to 1×10^{-3} M, was added to the assay mixture and the effect on the reaction rate determined. In another experiment, IdA at these same concentrations was preincubated with aldehyde oxidase at 37°C for 15–30 minutes prior to assay for enzymic activity. IdA exhibited no inhibition under either condition.

The capacity of IdA to function as a substrate for this enzyme was determined in the following assay mixture: IdA (1×10^{-5} to 1×10^{-3} M), 2,6-dichlorophenolindophenol (DCIP) (25 μg/ml), sodium phosphate buffer (0.03 M, pH 7.5), and enzyme. The reaction rate was determined by monitoring the rate of reduction of the electron acceptor (DCIP) at 600 nm using a Guilford recording spectrophotometer.

RESULTS

Excretion Studies

Data presented in figure 2 demonstrate that after administration of ^{14}C-IdA (iv to monkeys and dogs; ip to mice and rats) the majority of radioactivity is excreted primarily via the urinary route in each of these species. The dose for monkeys and dogs (10 mg/kg) was the highest nontoxic dose established by toxicology studies (10) and the dose for mice (180 mg/kg) was the optimal daily ip therapeutic dose determined for L1210 leukemia.[8] For rats, dogs, and mice, the excretion curves were hyperbolic, with a large portion of the radioactivity being excreted during the first few hours after administration. Urinary excretion appeared to be somewhat slower in mon-

[8]See footnote 7.

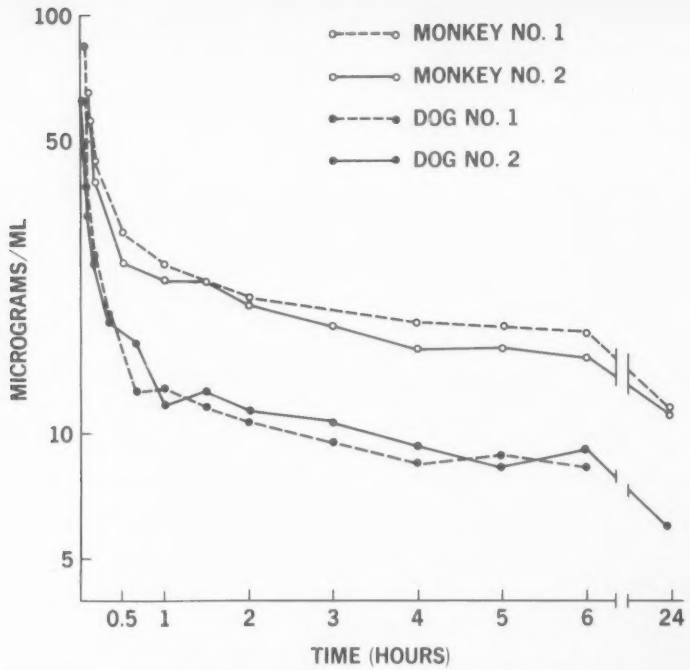

FIGURE 3.—Plasma levels of IdA determined from plasma radioactivity following iv injection of ^{14}C-IdA (10 mg/kg) in dogs and monkeys.

keys, although the total amount excreted in 24 hours was similar to that in the other species studied. In contrast, when ^{14}C-IdA was administered orally to mice, < 10% of the radioactive dose appeared in the urine. Table 1 summarizes the urinary and fecal excretion of radioactivity in mice receiving ^{14}C-IdA (180 mg/kg) orally, sc, iv, or ip. There is little difference in the urinary and fecal elimination pattern after sc, iv, or ip administration. However, when IdA was administered orally, most of the radioactivity was recovered in the feces and only a small fraction appeared in the urine. Following ip administration of ^{14}C-IdA to mice at doses of 90 and 360 mg/kg, the percentage of the dose excreted at 1,

TABLE 1.—Excretion of ^{14}C after iv, sc, ip, or oral administration of ^{14}C-IdA to mice*

Time (hrs) after administration	Excretion							
	iv		sc		ip		oral	
	%	Cumulative %	%	Cumulative %	%	Cumulative %	%	Cumulative %
				Urine				
1	42.5†	42.5	47.3	47.3	19.7	19.7	1.6	1.6
2	4.1	46.6	13.9	61.2	23.8	43.5	2.8	4.4
4	7.4	54.0	3.5	64.7	9.3	52.8	0.9	5.3
6	1.8	55.8	0.3	65.0	0.1	52.9	1.2	6.5
24	14.1	69.9	14.4	79.4	10.3	63.2	2.8	9.3
				Feces				
24		0		0		0		69.7

*CDF$_1$ mice (23 g) were given ^{14}C-IdA (180 mg/kg); the urine and feces were collected for 24 hours and analyzed for radioactive content as outlined in the Materials and Methods section. Each value is the average of 3 mice and expressed as percent of dose administered and cumulative percent.

†Values obtained at 1 hr were within 10% of the average indicated.

TABLE 2.—Excretion of ^{14}C following ip administration of ^{14}C-IdA to rats*

Time (hrs) after administration	Excretion in—			
	Urine		Feces	
	%	Cumulative %	%	Cumulative %
1	28.2†	28.2	—	—
2½	19.16	47.36	—	—
4	2.68	50.04	—	—
6	3.90	53.94	—	—
24	7.79	61.73	1.52	1.52
48	4.26	65.99	2.23	3.75
72	1.96	67.95	0.97	4.72
96	1.25	69.20	0.43	5.15
168	2.04	71.24	0.86	6.01

*Sprague-Dawley rats were given ^{14}C-IdA ip (40 mg/kg) and their urine and feces collected and assayed for ^{14}C at the indicated intervals. Values are averages of 3 rats and expressed as percent of dose administered and cumulative percent.
†Values obtained at 1 and 2.5 hrs were within 10% of the average indicated.

2, 4, 6, and 24 hours was similar to that observed with 180 mg/kg (table 1).

Table 2 summarizes the excretion of radioactivity in feces and urine for 7 days after ip administration of ^{14}C-IdA (40 mg/kg) to rats. Although most of the radioactivity was excreted in the urine within the first 24 hours, radioactivity could still be detected 7 days later in urine and feces. However, only a small fraction of the dose was eliminated in feces (6%). Radioactivity appearing in the feces is due to biliary excretion, since rats with cannulated bile ducts eliminated 6.9% of a 40-mg/kg iv dose in 24 hours.

Tissue Distribution

The data in figure 3 show the plasma levels of ^{14}C-IdA following iv administration to dogs and monkeys. In each of these species the curves depicting total ^{14}C-IdA are multiphasic with an initial rapid component over the first 30 minutes (half-life = 12–18 mins), followed by a much slower decline. The values obtained after 24 hours indicate only a slight decrease from those observed at 6 hours. Plasma radioactivity was followed for 28 days in dogs receiving 50 mg/kg iv and the long phase of the multiphasic curve of plasma disappearance had a half-life of approximately 3 days.

Treatment of plasma samples with cold 10% TCA revealed that a portion of the radioactivity was TCA insoluble and that this fraction of the total radioactivity increased with time after administration. These results are depicted in figure 4 using data obtained from dogs, although the same phenomenon occurred with the other species studied. As illustrated in figure 4 the portion of radioactivity that was TCA insoluble approached the total radioactivity curve and after 24 hours nearly all of the

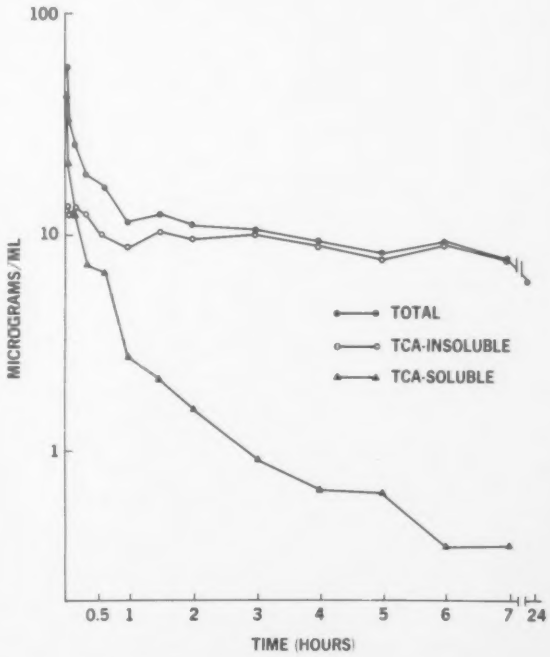

FIGURE 4.—TCA-soluble, TCA-insoluble, and total IdA concentrations in plasma determined from radioactivity following iv injection of ^{14}C-IdA (10 mg/kg) in dogs. Plasma samples were treated with 10% TCA and processed as outlined in the Materials and Methods section. Each value is the average of experiments in 2 dogs.

radioactivity was TCA insoluble. The radioactivity in the 24-hour plasma sample did not dissociate from protein even when a 2-ml sample was dialyzed against 3 liters of 0.01 M sodium phosphate buffer (pH 7.2) for 24 hours. However, treatment of the same sample with trypsin (1 mg/ml) for 16 hours at

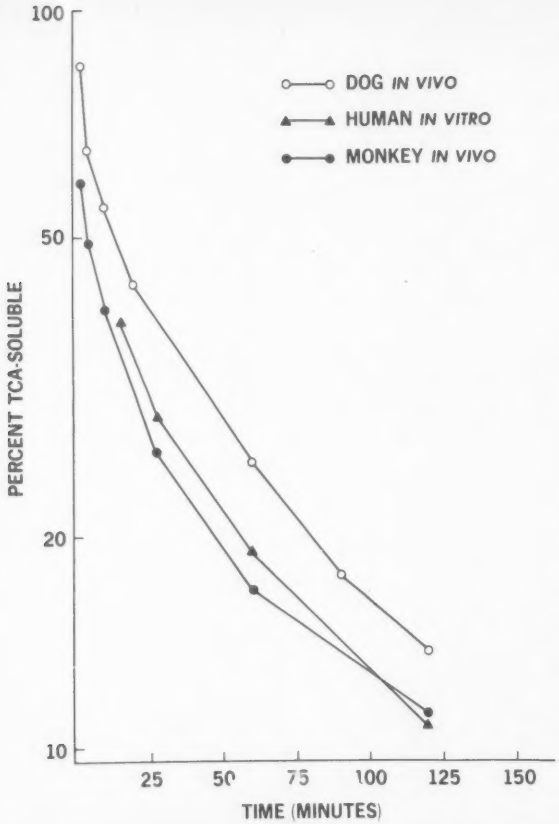

FIGURE 5.—Time dependence of in vitro and in vivo loss of TCA-soluble radioactivity. Data for dogs and monkeys taken from experiments shown in fig 4 and expressed as percent of TCA-soluble ^{14}C-IdA. Human plasma was incubated with ^{14}C-IdA (200 μg/ml) at 37°C and the percent of TCA-soluble ^{14}C-IdA was determined at the indicated time intervals.

37°C rendered the radioactivity TCA soluble and dialyzable. This indicated that the radioactivity was protein bound and the drug-protein complex extremely stable under physiologic conditions. Cerebrospinal fluid (CSF) samples taken periodically from monkeys and dogs following iv injection revealed that the amount of radioactivity entering the CSF was only slightly above background levels. This was expected since IdA only very slightly partitioned into heptane or chloroform (< 0.02%) from an aqueous or buffered saline solution.

The data in table 3 show concentrations of radioactivity (expressed as micrograms of IdA per gram of tissue) in several tissues at various intervals after ip administration to mice at a dose of 180 mg/kg. There does not appear to be preferential localization of radioactivity in any organ. In table 4, tissue concentrations of radioactivity are compared in mice 24 hours after receiving ^{14}C-IdA ip at doses of 90, 180, and 360 mg/kg. Again, there appears to be no preferential uptake of radioactivity by any organ. Furthermore, the radioactivity in these 24-hour tissue homogenates was insoluble in 10% TCA or 95% ethanol and was nondialyzable.

Metabolism Studies

A series of in vitro experiments were performed to elucidate the nature of the interaction of IdA with biologic material. IdA interacts with both human plasma and bovine serum albumin (BSA) in vitro forming a complex that is TCA insoluble. The data in figure 5 show the rate of loss of TCA solubility when IdA was incubated in vitro with human plasma compared to that observed in vivo in dogs and monkeys following iv injection. The concentration of IdA used in the in vitro portion of the experiment was comparable to the plasma concentration of IdA obtained in these animals immediately after iv injection. The data shown in figure 5 demonstrate that as IdA is exposed to human plasma in vitro, the percentage of IdA that remains TCA soluble decreases at a rate similar to that observed in vivo following iv administration to dogs and monkeys.

IdA is not a substrate for rabbit liver aldehyde oxidase and does not inhibit this enzyme at concentrations up to 1×10^{-3} M when assayed as outlined in the Materials and Methods section. Furthermore, chromatographic analysis of the urine from ^{14}C-IdA-treated animals indicated that the radioactivity was present as unchanged IdA and as complexes of IdA with urine constituents. Thus, chromatography of urine from ^{14}C-IdA-treated animals on silica gel plates developed in an ethyl acetate:ethanol:water (10:10:3) system yielded a series of radioactive spots that were nearly identical in pattern to that obtained when ^{14}C-IdA was added to normal urine and chromatographed in the same system. The predominant radioactive spot appearing in the urine of ^{14}C-IdA-treated animals had the same R_f value as an IdA-urea complex. Acidification of the urine with 1 N HCl and subsequent chromatography in a butanol:acetic acid:water (4:1:1) system resulted in a disruption of the complexes and the radioactivity migrated as unchanged IdA. This is consistent with a Schiff-base reaction between the aldehyde groups of IdA and amino groups of urinary constituents. The interaction of IdA with proteins and low molecular weight compounds is the subject of the following paper in this issue (4).

TABLE 3.—Distribution of ^{14}C in mouse tissues*

Time (mins)	Distribution (μg IdA/g of tissue) in—†						
	Liver	Lung	Kidney	Spleen	Small intestine	Large intestine	Bladder
5	382 ± 16.2†	262 ± 5.5	491 ± 51.2	607 ± 14.7	396 ± 28.1	373 ± 47.7	916 ± 59.0
10	326 ± 3.2	217 ± 8.2	469 ± 40.2	347 ± 9.3	297 ± 23.0	342 ± 30.9	1306 ± 49.5
30	168 ± 16.1	135 ± 4.3	270 ± 19.1	171 ± 10.1	189 ± 19.8	143 ± 2.5	358 ± 34.8
60	90.3 ± 9.1	91.8 ± 4.3	125 ± 2.7	82 ± 2.3	134 ± 20.0	106 ± 9.3	203 ± 51.6
240	57.8 ± 5.1	60.8 ± 0.65	83.6 ± 6.9	62.8 ± 9.4	126 ± 5.3	84 ± 11.9	149 ± 24

*CDF_1 mice were injected with ^{14}C-IdA ip (180 mg/kg, 4 mice/group) and killed at the indicated time intervals.
†Values are the mean ± SE for 4 mice.

TABLE 4.—Distribution of ^{14}C in mouse tissues 24 hrs after ip injection of ^{14}C-IdA*

Dose (mg/kg)	Distribution (μg IdA/g of tissue) in—						
	Liver	Lung	Kidney	Spleen	Small intestine	Large intestine	Bladder
360	96.25 ± 12.74†	115.75 ± 14.06	112.80 ± 11.81	141.80 ± 29.10	177.00 ± 13.47	214.00 ± 23.50	151.30 ± 19.50
180	33.50 ± 2.22	51.75 ± 0.63	61.00 ± 2.97	40.25 ± 1.32	57.25 ± 4.09	149.80 ± 21.22	82.00 ± 3.63
90	17.50 ± 1.55	38.00 ± 1.58	39.75 ± 3.25	26.25 ± 3.28	29.75 ± 3.30	68.00 ± 21.35	31.67 ± 3.53

*CDF_1 mice were injected with ^{14}C-IdA and killed 24 hours later.
†Values are the mean ± SE for 4 mice.

DISCUSSION

The pharmacologic disposition of IdA is greatly influenced by the relative extent of interaction with protein macromolecules versus low molecular weight compounds. Following iv or ip administration of ^{14}C-IdA, most of the IdA is eliminated in the urine where it is found as complexes with urine constituents (mainly urea). These complexes can be dissociated with acid and IdA recovered unchanged. Failure to detect metabolites chromatographically agrees with a previous study (11) in which a chemical assay for IdA was employed to show that all of the radioactivity appearing in the urine of dogs injected with ^{14}C-IdA could be recovered as unchanged IdA following a procedure designed to disrupt IdA-small molecule complexes. In the present study it was found that IdA is not a substrate for aldehyde oxidase in vitro. Therefore, even though IdA has two aldehyde groups that would appear to be quite vulnerable to oxidation, in fact no such oxidation occurs in vivo or with the appropriate enzyme in vitro. One explanation for this finding may be that IdA exists as the hemi-acetal or hemi-aldehyde form as reported for other dialdehydes (1), and this configuration might be resistant to enzymatic oxidation. An alternative explanation for the lack of metabolism is the very rapid reaction of IdA with proteins and small molecules which would protect the aldehyde groups from such oxidation in vivo.

The multiphasic plasma radioactivity curves observed in all species resulted from the interaction of IdA with plasma proteins. Following iv administration, plasma radioactivity declined with an initial half-life of 12–18 minutes followed by a slower component with a half-life of approximately 3 days. At all time intervals total plasma radioactivity was the sum of TCA-soluble and TCA-insoluble radioactivity, the portion of radioactivity that was TCA insoluble increasing with time after injection. A similar rate of decreasing percentage of TCA-soluble radioactivity could be duplicated in vitro using human plasma and a concentration of IdA that was equivalent to that determined immediately after iv injection in dogs. The complex of IdA with plasma or cellular proteins was stable and did not dissociate upon dialysis, TCA precipitation, or Sephadex G-200 gel filtration. There was no preferential localization of ^{14}C-IdA in any organ in mice at various times after ip administration. There was essentially no penetration of IdA into the CSF of monkeys and dogs as predicted by the extremely low solubility of IdA in organic solvents and its unfavorable partition coefficient.

The specificity of the interaction of IdA with proteins and low molecular weight compounds and the effect of these interactions on the cytotoxicity of

IdA are the subject of the following paper in this issue (4).

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Protein Cross-Linking Properties of the Antitumor Agent Inosine Dialdehyde (NSC-118994)[1,2]

Richard L. Cysyk* and Richard H. Adamson[3,4]

SUMMARY

Inosine dialdehyde (IdA), a new antitumor agent presently undergoing clinical evaluation in man, possesses two aldehyde groups that form stable complexes with a variety of biologic molecules containing amino groups. Complex formation of IdA with lysine, glycine, histidine, or bovine serum albumin (BSA) greatly reduces the cytotoxicity of IdA against L1210 leukemia in vitro. Complexes of IdA and BSA exhibit molecular weights ranging from 69,000 to > 800,000 as determined by Sephadex G-200 gel filtration, indicating that both aldehyde groups of IdA are functional and can cross-link protein molecules. The cross-linking of plasma proteins and the cross-linking of glycine to BSA were also observed. No interaction of IdA with nucleic acids, nucleic acid bases, or nucleosides was detected. The dialdehyde derivatives of other nucleosides also possessed cross-linking properties.

[Cancer Treat Rep 60:563–570, 1976]

Inosine dialdehyde (IdA), the periodate-oxidation product of inosine, is highly active against several transplantable murine tumors including L1210 and P388 leukemias and a number of variants of L1210 resistant to commonly used cancer chemotherapeutic agents (1).[5] IdA is now undergoing clinical evaluation in man (2). In a previous study of the pharmacologic disposition of IdA (3), we found that IdA interacted with plasma and cellular proteins and with low molecular weight constituents of urine and plasma forming complexes that were stable under physiologic conditions. The results from that report indicated that the relative extent of interaction of IdA with protein versus low molecular weight molecules determined the pharmacologic disposition of IdA. In the present study we determined the effects of such interactions on the cytotoxicity of IdA and investigated the chemical interaction of IdA with proteins and low molecular weight compounds.

MATERIALS AND METHODS

^{14}C-[8]-IdA, synthesized by Stanford Research Institute, and IdA were obtained from the Drug Research and Development Program, DCT, NCI. Crystalline bovine serum albumin (BSA) and calf-thymus DNA were purchased from Schwarz/Mann, Orangeburg, NY; Sephadex G-200 was purchased from Pharmacia Fine Chemicals, Inc, Piscataway, NJ; and ^{14}C-[2]-glycine was obtained from Amersham-Searle, Arlington Heights, Ill. Rabbit muscle aldolase was purchased from Worthington Biochemical Corp, Freehold, NJ.

Cytotoxicity Studies

In vitro cytotoxicity experiments were performed with L1210 leukemia cells maintained in static culture in RPMI 1630 medium with 20% fetal calf serum. A stock culture was diluted to yield 10^5 cells/ml and a total volume of 5 ml/bottle. Solutions of IdA or other mixtures to be tested were passed through a 0.45μ millipore filter and a volume of 0.1 ml was added to each bottle. The cells were counted by means of a Coulter counter at 24 and 48 hours.

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[2]Inosine dialdehyde: diglycolaldehyde, α-(hydroxymethyl)-α'-(6-hydroxy-9*H*-purin-9-yl)-.

[3]Laboratory of Chemical Pharmacology (LCHPH), Experimental Therapeutics Program (ETP), Division of Cancer Treatment (DCT), National Cancer Institute (NCI), Bethesda, Md.

[4]The technical assistance of Miss Mary Ellen Ensminger and Mrs. Ovella Ayers is appreciated.

[5]Cysyk RL, and Adamson RH. Antitumor properties of inosine dialdehyde. In preparation.

**Reprint requests to*: Dr. Richard L. Cysyk, LCHPH, ETP, DCT, NCI, Bldg 10, Rm 6N119, National Institutes of Health, Bethesda, Md 20014.

Interaction With Low Molecular Weight Compounds

Complexes of IdA with urine constituents (mainly urea, but other small molecules also) could be separated from free IdA by thin-layer chromatography (3). A series of compounds were tested for their reactivity with IdA by incubating ^{14}C-IdA (20 mM) with the compound (20 mM) in 0.05 M sodium phosphate buffer (pH 7.4) for 60 minutes at 37°C. A 2-μl volume of the reaction was applied to a thin-layer silica gel plate prewashed with ethyl acetate:ethanol:water (10:10:3) and developed in the same system. In this system IdA had an R_f value of 0.62 while most other compounds tested migrated only slightly from the origin. Complexes of IdA and other compounds, when formed, had intermediate R_f values. Amino acids were detected by ninhydrin reaction and other molecules by ultraviolet absorbency. ^{14}C-IdA and complexes with ^{14}C-IdA were detected by autoradiography and quantitated by removing the spots and determining radioactivity. Dissociation of the complexes occurred when the solution was made acid with 1 N HCl and chromatographed on thin-layer silica gel plates in a butanol:acetic acid:water (4:1:1) system. The interaction of IdA with protein was determined by trichloroacetic acid (TCA) precipitation as described previously (3).

Gel Filtration Studies

The effect of IdA and other diglycolaldehyde derivatives on the Sephadex G-200 elution pattern of BSA, plasma, or DNA was determined by reaction in 0.05 M phosphate buffer (pH 7.4) for 60 minutes at 37°C and application of 1 ml of the reaction mixture to a Sephadex G-200 column (2.5 × 100 cm) previously equilibrated with the reaction buffer and then eluted with the same. Fractions were collected dropwise (3 ml/fraction, 0.4 ml/min) using an LKB fraction collector and analyzed for protein by the method of Lowry et al (4), for DNA by 260-nm absorbency, or for radioactivity by addition of 0.1 ml of each fraction to 18 ml of scintillation fluid [6.0 g 2,5-diphenyloxazole, 0.2 g 1,4-bis-2-(5-phenyloxazolyl)-benzene, 1400 ml toluene, 600 ml methanol] and counting in a Beckman LS-230 scintillation counter.

Enzyme Assays

The assay for rabbit muscle aldolase was a modification of the original assay described by Jagannathan et al (5). The assay mixture contained fructose-1,6-diphosphate (4 mM), hydrazine sulfate (2.4 mM), EDTA (0.06 mM), sodium phosphate buffer (20 mM, pH 7.5), and enzyme (0.02 unit). The reaction was carried out at 25°C and the increase in 240-nm absorbency followed with a Guilford recording spectrophotometer. A blank cuvette contained all of the reactants except the enzyme and was subtracted from those reactions containing enzyme.

Rabbit liver aldehyde oxidase was purified according to the method of Rajagopalan et al (6) and assayed by following the conversion of methotrexate to 7-hydroxymethotrexate at 37°C as described by Johns et al (7). The change of 340-nm absorbency was determined with a Guilford recording spectrophotometer.

Escherichia coli RNA polymerase was assayed by the method of Richardson (8). Calf-thymus DNA was used as the template and the incorporation of ^{14}C-adenosine-5′-triphosphate into TCA-insoluble material was measured using the filter-paper disc method of Bollum (9).

Interaction With Red Blood Cell Membranes

The interaction of ^{14}C-IdA with red blood cell membranes was determined by the method of Kimball et al (10). Human red blood cells were washed twice with phosphate-buffered saline by alternate suspension and centrifugation. A volume of 1 ml of the red blood cell pack was diluted with an equal volume of phosphate-buffered saline and incubated with 1.59 mg ^{14}C-IdA (665 cpm/μg) for 30 minutes at 37°C. Cells were centrifuged and washed repeatedly in 10-ml volumes of cold phosphate-buffered saline until wash samples were free of radioactivity. The cells were then lysed in 10 ml distilled water and the lysate was centrifuged at 16,000 × g for 60 minutes. Pellets were washed and centrifuged until the wash was free of radioactivity. The pellets were then dissolved in NCS tissue solubilizer and the content of radioactivity was determined.

RESULTS

Interaction With Small Molecules

In the preceding paper in this issue (3) it was noted that IdA interacts with cellular and plasma proteins and with low molecular weight constituents of urine. Complexes of IdA and protein were TCA insoluble and nondialyzable. A series of low molecular weight compounds were tested for their reactivity with IdA as described in the Materials and Methods section. This procedure was capable of detecting complexes containing as little as 1% of the total IdA. In those cases where complexes were observed, 20%–50% of the total IdA was contained in the complexes. The results in table 1 show that

TABLE 1.—Reactivity of IdA with various molecules*

Interaction	No interaction
All amino acids possessing a primary amino group	Proline, hydroxyproline, *N*-acetylcysteine
BSA, glutathione	*N*-acetylglycine, *N*-acetylhistidine, *N*-acetylarginine
Polyamines (spermidine, spermine, cadaverine, putrescine)	DNA, RNA
Urea	Nucleic acid bases, nucleosides, nucleotides, deoxynucleosides, deoxynucleotides
N-acetyllysine	
D-Glucosamine	Glucose

*^{14}C-IdA (20 mM) was incubated with the compound (20 mM) in 0.05 M sodium phosphate buffer (pH 7.4) for 60 mins at 37°C. A 2-μl sample was applied to a thin-layer silica gel plate prewashed and developed with ethyl acetate:ethanol:water (10:10:3). Complexes were detected by radioactivity measurements. Interaction with BSA, DNA, and RNA was examined by TCA precipitation and Sephadex G-200 filtrations. Details of experimental procedure are in the Materials and Methods section.

IdA forms complexes with all amino acids containing a primary amino group. No complex is formed when the primary amino group is acetylated (eg, *N*-acetylglycine, *N*-acetylarginine, *N*-acetylhistidine, and *N*-acetylcysteine) or with the secondary amino groups of proline or hydroxyproline. Interaction does occur with *N*-acetyllysine which has an ϵ-amino group, indicating that IdA could react with lysine side chains within a protein molecule.

No complexes could be detected between IdA and nucleic acids, nucleosides, nucleotides, or nucleic acid bases even though some of these possess amino groups. Complexes were formed with polyamines (spermidine, spermine, cadaverine, and putrescine). The interaction of IdA with phenylhydrazine has been utilized as an assay for IdA in biologic fluids (11). IdA also interacts with D-glucosamine but not with glucose.

Effect of Small Molecules on IdA Cytotoxicity

Since IdA interacts rapidly with biologic material in vivo and in vitro it was necessary to determine the effect of such interactions on the cytotoxicity of IdA. The results in table 2 show the effects of several compounds on the in vitro cytotoxicity of IdA. In these experiments IdA was incubated with the compound at the concentrations indicated for 60 minutes at 37°C as described in the Materials and Methods section. A volume of 0.1 ml of the reaction mixture was added to 5.0 ml of L1210 cells and the cells were counted at 24 and 48 hours. The results in table 2 show that prior incubation of IdA with BSA, histidine, lysine, or glycine reduced the subsequent in vitro cytotoxicity of IdA. These effects are exhibited even after a considerable dilution of the reaction mixture into the cell culture medium, indicating that the complexes formed were stable and did not dissociate upon dilution. Urea did not reduce the cytotoxicity of IdA even though it is capable of forming complexes with IdA both in vivo (3) and in vitro, suggesting a difference in the stability of this complex as compared with complexes formed between IdA and amino acids or peptides. Preincubation of ^{14}C-IdA (0.34 mM) with lysine, glycine, glutathione, or spermidine (each at 4.27 mM) for 60 minutes at 37°C inhibited the subsequent interaction of ^{14}C-IdA with BSA to the extent of 43%–80%. In contrast, urea (4.7 mM) had little effect (5% inhibition) on the interaction of IdA with BSA.

TABLE 2.—Effect of various compounds on the cytotoxicity of IdA to L1210 cells*

Compounds	% inhibition of L1210 growth
Lysine (1.71 mM)	32
Glycine (1.71 mM)	34
Histidine (4.27 mM)	33
Urea	
4.27 mM	75
1.71 mM	76
0.85 mM	71
BSA (1 mg/ml)	24
Control (IdA alone)	83

*IdA (0.34 mM) was incubated alone or with the compounds indicated for 60 mins at 37°C in 0.05 M sodium phosphate buffer (pH 7.4). A sample of each incubation mixture was added to a suspension of L1210 cells and the inhibition of cell growth determined after 24 hrs of incubation. Under these conditions IdA alone inhibited cell growth by 83%. Values are from 2 separate experiments, each employing duplicates for each determination. Details of the experimental procedures are in the Materials and Methods section.

Protein Cross-Linking Properties of IdA

IdA has two aldehyde groups. To determine if both groups are capable of reacting with amino groups the product of the IdA-BSA complex was examined by Sephadex G-200 filtration. If both aldehyde groups of IdA are capable of interaction, then cross-linking of BSA molecules might occur and the product would exhibit an increased molecular weight in this system. That this does occur is shown in figure 1. A mixture of BSA (10 mg/ml) and high molecular weight blue dextran was chromatographed on a Sephadex G-200 column, as described in the Materials and Methods section, to determine the void volume and the R_f value of BSA. The R_f value for BSA was 0.56, as previously reported (12). ^{14}C-IdA (10 mg/ml) and BSA (10 mg/ml) were reacted at 37°C for 180 minutes in 0.05 M sodium phosphate buffer (pH 7.4). A volume of 1.0 ml was placed on the column and eluted with 0.05 M sodium phosphate buffer (pH 7.4), and the fractions were analyzed for protein and radioactivity. After reaction with IdA, BSA is found in fractions corresponding to a molecular weight of 69,000 and greater. A significant amount of protein eluted in the void volume indicating a molecular weight of at least 800,000 (12). Assay of each fraction for radioactivity indicated IdA to be associated with protein and the elution pattern of radioactivity compared closely with that of the protein. The final peak of radioactivity is unreacted IdA. The number of molecules of IdA bound per molecule of BSA was constant throughout the protein fractions and was found to be 45 ± 5. This suggests that the relative extent of intramolecular versus intermolecular interaction determines the magnitude of the molecular weight increase. The cross-linking is presumed to be between the ε-amino groups of lysine residues within the protein molecule because IdA reacts with *N*-acetyllysine but not with other *N*-acetylated amino acids. The terminal amino acid of each BSA protein chain would also be capable of interacting with IdA.

To further examine the bifunctional reactivity of IdA, ^{14}C-glycine was added to a mixture of BSA and IdA to determine if IdA could cross-link glycine to BSA. Following incubation of ^{14}C-glycine (10 mg/ml), BSA (10 mg/ml), and IdA (10 mg/ml), a volume of 1.0 ml of the mixture was chromatographed on Sephadex G-200 as before. The data in figure 2 show that ^{14}C-glycine and BSA (no IdA) are completely separated by Sephadex G-200 filtration. However, in the presence of IdA, there was an increase in the molecular weight of the protein and an attachment of ^{14}C-glycine to the protein fractions. Again, the radioactivity followed closely the protein elution profile. These results indicate that IdA can cross-link low

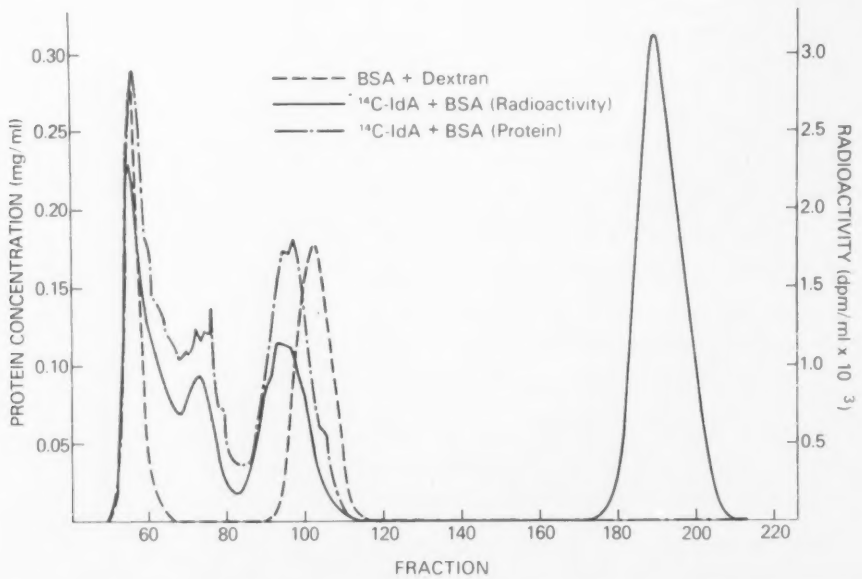

FIGURE 1.—Effect of IdA on the Sephadex G-200 elution pattern of BSA. BSA (10 mg/ml) was incubated with either high molecular weight blue dextran or ^{14}C-IdA (10 mg/ml) in 0.05 M sodium phosphate buffer (pH 7.4) for 180 mins at 37°C and samples of the reaction mixture were chromatographed as described in the Materials and Methods section.

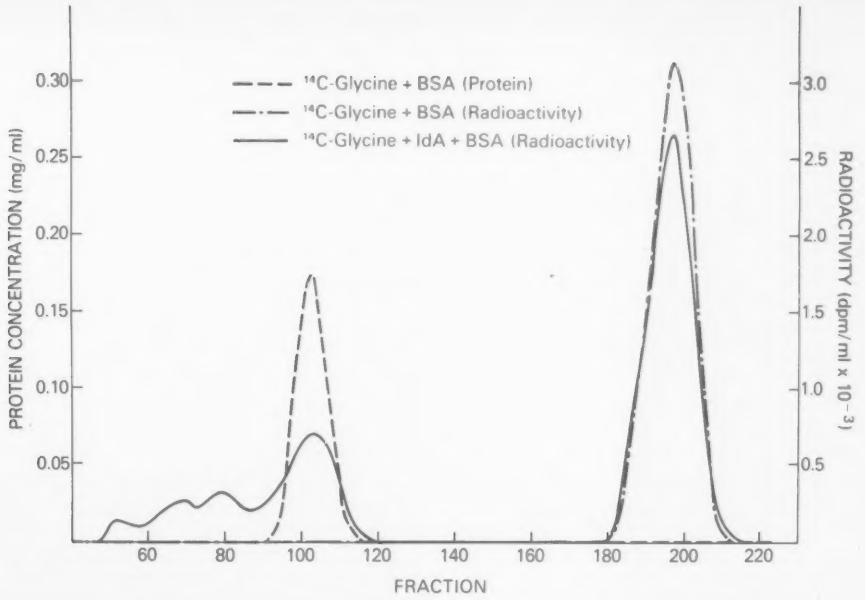

FIGURE 2.—Sephadex G-200 elution profile of ^{14}C-glycine following reaction with BSA or with a mixture of BSA and IdA. ^{14}C-glycine (10 mg/ml) was incubated with either BSA (10 mg/ml) or a mixture of BSA (10 mg/ml) and IdA (10 mg/ml) in 0.05 M sodium phosphate buffer (pH 7.4) for 180 mins at 37°C and samples of the reaction mixture were chromatographed as described in the Materials and Methods section.

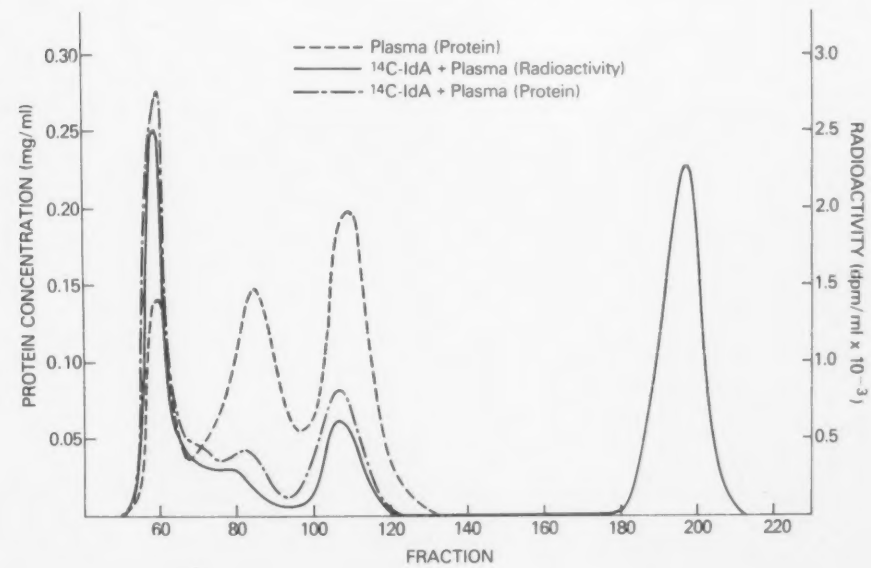

FIGURE 3.—Effects of IdA on the Sephadex G-200 elution profile of human plasma. Human plasma was incubated alone or with ^{14}C-IdA (10 mg/ml) for 180 mins at 37°C and chromatographed as described in the Materials and Methods section.

molecular weight compounds to proteins in addition to cross-linking protein macromolecules. Accordingly, some of the IdA molecules attached to cross-linked protein molecules possess aldehyde groups capable of interacting with small molecules.

The effect of IdA on the Sephadex elution pattern of human plasma is shown in figure 3. When incubated with ^{14}C-IdA (10 mg/ml) for 180 minutes at 37°C there is a general increase in the molecular weight of the protein fractions of human plasma with a significant amount of protein eluting in the void volume. ^{14}C-IdA is uniformly distributed throughout the protein fractions and the protein elution pattern follows closely the profile of radioactivity. This interaction of IdA with plasma proteins might account for the altered thrombin times of patients receiving IdA (2).

The periodate-oxidation products of adenosine, cytosine, and methylribose possess antitumor activity against mouse L1210 leukemia (13). When these compounds were each incubated with BSA and the reaction mixture was chromatographed on Sephadex G-200, the protein elution profiles were similar to that obtained from reacting IdA with BSA. Thus, the cross-linking activity of these compounds appears to be independent of the moiety attached to the diglycolaldehyde portion of the molecule. In contrast, the bisulfite addition product of the periodate-oxidation product of *O*-methylribose had no effect on the Sephadex G-200 chromatographic pattern of BSA.

When ^{14}C-IdA was incubated with calf-thymus DNA for 180 minutes at 37°C and the mixture chromatographed on Sephadex G-200, no radioactivity was detected in those fractions containing DNA and there was no alteration in the elution pattern of DNA. This agrees with data in table 1 showing no interaction of IdA with nucleic acid constituents.

The stability of the IdA-protein complexes formed at pH 7.2 suggested that ^{14}C-IdA might possibly be used to label lysine residues located at the active sites of enzymes. Evidence suggests that rabbit muscle aldolase possesses a lysine at its active site (14) and amino acid analysis of this enzyme indicates that the amino-terminal amino acids of each of its two polypeptide chains are proline residues. No interaction occurs between IdA and proline, so any binding between IdA and aldolase would occur through lysine residues. However, it was found that concentrations of IdA as high as 1×10^{-3} M had no effect on aldolase activity when assayed as outlined in the Materials and Methods section. Furthermore, incubation of IdA and aldolase for 2 hours at 25°C before addition of substrate had no effect on the enzymatic activity of aldolase. It was demonstrated previously (3) that IdA was not a substrate for rabbit liver aldehyde oxidase and did not inhibit the activity of this enzyme. Incubation of rabbit liver aldehyde oxidase with 1×10^{-3} M IdA for 30 minutes at 37°C did not decrease the activity of this enzyme. Also, *E. coli* RNA polymerase was not inhibited by 1×10^{-3} M IdA when assayed as outlined in the Materials and Methods section. These results indicate that either IdA is not interacting with critical lysine residues by virtue of the conformation of the protein molecule, or that if a reaction is occurring the physical environment at the active site is such that the complex formed is not a stable one and readily dissociates.

To investigate the possible interaction of IdA with cell membranes, ^{14}C-IdA was incubated with human red blood cells as outlined in the Materials and Methods section. After a 30-minute exposure, 23.2% of the added IdA was taken up into the cells. Since there were no small molecules present in the incubation medium capable of reacting with IdA, the uptake of ^{14}C-IdA was that of free IdA. After the cells were lysed and washed repeatedly with distilled water, the membrane fraction was found to contain 0.23% of the total IdA added. IdA attached to red blood cell membranes could explain the positive Coomb's reaction exhibited by patients receiving IdA (15), provided the membranes were cross-linked with plasma proteins via the IdA molecule.

DISCUSSION

Previous studies (3) in experimental animals demonstrated that IdA is not metabolized to any detectable extent and that the pharmacologic disposition of IdA is determined by the chemical interaction of IdA with plasma and cellular materials. IdA is excreted in the urine as complexes with low molecular weight compounds and IdA remaining in plasma and tissues is attached to proteins and does not dissociate upon dialysis or TCA precipitation. In the present study the interaction of IdA with proteins and low molecular weight compounds was examined to determine the specificity of the reaction and the effect of such interactions on the cytotoxicity of IdA.

IdA forms complexes with a variety of small molecules possessing amino groups. The interaction with amino acids requires a primary amino group. No interaction occurs with proline, with hydroxyproline, or when the primary amino group is acetylated (eg, *N*-acetylglycine, *N*-acetylhistidine, *N*-acetylarginine, *N*-acetylcysteine). Interaction does occur with the ϵ-amino group of *N*-acetyllysine indicating that such a reaction could occur with lysine residues within a protein molecule. IdA does not react with purines or pyrimidines even though some

of these possess amino groups. Complexing of IdA with lysine or glycine reduced the in vitro cytotoxicity of IdA. Urea, in contrast, complexes with IdA but did not reduce IdA cytotoxicity, indicating a difference in the reversibility of the two types of complexes. The reduction in toxicity of IdA by these amino acids suggests their possible use in controlling the clinical toxicity of IdA. Glycine, lysine, spermidine, and glutathione inhibit the interaction of IdA with BSA, whereas urea had no effect.

Interaction of IdA with BSA results in an increase in the molecular weight of BSA ranging from 69,000 to > 800,000 as determined by Sephadex gel filtration, indicating extensive intermolecular cross-linking of BSA molecules by IdA. Thus, both aldehyde groups on IdA are functional and can participate in reactions with primary amino groups of two separate protein molecules. The cross-linking most likely occurs between ε-amino groups of lysine residues within the protein molecule since IdA reacts strongly with *N*-acetyllysine but not with *N*-acetylarginine or *N*-acetylhistidine. There are 57 lysine residues per BSA molecule (16). The number of IdA molecules bound per BSA molecule was constant at 45 throughout the multiple peaks of the Sephadex G-200 elution pattern. Therefore, not all of the lysine residues were bound to IdA. Many of the IdA molecules attached to BSA possessed an unreacted aldehyde group since ^{14}C-glycine could be attached to the IdA-BSA complex. Incubation of human plasma with IdA resulted in an increase in the molecular weight of plasma proteins. Such an interaction with plasma proteins might explain the increased thrombin times reported for patients receiving IdA (2). Also, cross-linking of plasma proteins with red blood cell membranes could result in the positive Coomb's reaction exhibited by these patients (15). In the present study, IdA was found attached to red blood cell membranes following incubation with human red blood cells in vitro. Also, IdA passes freely into the cell as free IdA and can interact with cellular proteins.

The periodate-oxidation products of adenosine, cytosine, and methoxyribose are active against mouse L1210 leukemia (13). In the present study they were found to produce cross-linking of BSA molecules as does IdA. No cross-linking was found when the aldehyde groups of the periodate-oxidation product of methoxyribose were altered by bisulfite addition. The chemical reactivity of the periodate-oxidation products with proteins would appear to be nonspecific and cytotoxic due to a general inactivation of cellular proteins. Enzyme inactivation by periodate-oxidation products of other nucleosides has been reported (17–20). The oxidation product of 6-methylthiopurine riboside has been shown to inhibit DNA-dependent RNA polymerase (17), thymidylate kinase (18), and bovine pancreatic ribonuclease A (19). Ribonucleotide reductase is inhibited by the periodate-oxidation products of adenosine, adenosine-5′-monophosphate, and adenosine-5′-triphosphate (20), although the relative effects of these compounds on the catalytic and allosteric sites were not clear. The degree of specificity imparted on these molecules by the purine base is unclear since pyridoxal phosphate is also known to inhibit these enzymes by reacting with critical lysine residues (20). In our studies IdA did not inactivate *E. coli* RNA polymerase, rabbit liver aldehyde oxidase, or rabbit muscle aldolase, indicating that specificity of enzyme inactivation might be determined by the base attached to the diglycolaldehyde portion of the molecule. The lack of inhibition of enzyme activities by IdA could be a result of IdA not attaching to critical lysine residues because of protein conformation, or the conditions at those sites could favor dissociation of IdA from the complex. In addition to inactivating enzymes by reaction with critical lysine residues, it would seem reasonable to predict from the data presented here that cytotoxicity might also occur by cross-linking critical enzymes to cellular constituents.

In a study of the effect of the periodate-oxidation product of 6-methylthioinosine on DNA, RNA, and protein synthesis, it was reported that the utilization of glycine for protein synthesis was the most sensitive area of inhibition by this compound (21). The possible interaction of drug and glycine in this system predicted by our findings complicates the interpretation of such results. Future studies with periodate-oxidation products should use proline incorporation as an indicator of protein synthesis to obviate this complication.

IdA had no effect on the Sephadex G-200 elution profile of DNA. Also, no attachment of ^{14}C-IdA to DNA, RNA, or a number of nucleosides, nucleotides, and nucleic acid bases was detected. The bifunctional aldehydes glyoxal and malondialdehyde have been shown to interact with DNA and the nucleic acid bases cytidine and guanine (22). The lack of interaction of IdA with DNA molecules indicates that the spatial arrangement of the two aldehyde functions is of importance in determining the specificity of the interaction or the stability of the complex formed.

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Modulating Effects of Levamisole (NSC-177023) on Human Lymphocyte Response In Vitro[1,2,3]

J. Leonard Lichtenfeld,[4,*] Myles R. Desner,[4,5] Peter H. Wiernik,[4] and Michael R. Mardiney, Jr.[4]

SUMMARY

Human lymphocytes were cultured in vitro with multiple concentrations of antigen, mitogen, and allogeneic cells. The addition of levamisole to stimulated cultures in concentrations of 0.03–33 μg/ml significantly amplified the response of lymphocytes to these stimuli with little or no effect on control cultures. The combination of stimulus and levamisole concentration which produced amplification was unique for each individual culture. The inconsistent results found here and elsewhere may reflect instability of levamisole under conditions of the in vitro lymphocyte culture system.

[Cancer Treat Rep 60:571–574, 1976]

Clinical trials with levamisole, a low molecular weight orally administered antihelminthic, revealed that this drug improved the responses of some patients with advanced cancer to challenge with the contact allergen dinitrochlorobenzene (DNCB) (1,2). Other investigators reported that levamisole prolonged the disease-free interval and survival of mice with transplanted leukemia (3) and patients with lung cancer (4) or breast cancer (5) when the drug was administered following primary treatment with chemotherapy, surgery, or radiation respectively. Levamisole may also have therapeutic activity in a variety of nonmalignant conditions as well (6).

The mechanism of action of levamisole as an immune modulator has not been defined (6,7). Previous experience with lymphocytes cultured in vitro demonstrated the value of this system as a method to evaluate immune stimulators (8). This study describes similar experiments in which levamisole improved the response of lymphocytes to several types of immunologic stimuli including specific antigen (streptokinase-streptodornase [SKSD]), mitogen (phytohemagglutinin-M [PHA-M]), and allogeneic cells, indicating that this drug has a direct effect on the cell-associated immune system.

MATERIALS AND METHODS

Culture conditions and cell preparations.—Our techniques of lymphocyte culture have been previously described (9,10). Briefly, 3×10^6 responding leukocytes obtained from heparinized (20 units/ml) sedimented whole blood were cultured in RPMI 1640 media containing 15%–20% autologous plasma, 50 units/ml penicillin, 50 μg/ml streptomycin, and 300 μg/ml glutamine. All cultures were performed in triplicate in a final volume of 1.5 ml of media with various concentrations of soluble antigens, mitogens, or irradiated allogeneic leukocytes and incubated at 5°C from the horizontal in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. One-way mixed leukocyte cultures contained 1.5×10^6 responding cells and 1.5×10^6 irradiated (1000 rads) allogeneic stimulating leukocytes per culture. To evaluate thymidine incorporation, 2 microcuries of tritiated thymidine (specific activity, 1.9 curies/millimol; Schwarz/Mann, Orangeburg, NY) was added to mitogen-stimulated cultures on the 4th day and to leukocyte- and antigen-stimulated cultures on the 7th day. Cultures were incubated for an additional 4 hours and then washed with chilled normal saline. Tritiated thymidine incorporation was determined by standard methods of scintillation counting (9,10) and expressed as mean disintegrations per minute

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[2]Presented in part at the 58th annual meeting of the Federation of American Societies for Experimental Biology, Atlantic City, NJ, Apr 7–12, 1974.

[3]Levamisole: CAS reg. No. 16595–80–5; imidazo[2,1-*b*]thiazole, 2,3,5,6-tetrahydro-6-phenyl-, monohydrochloride. Supplied by Lederle Laboratories, Pearl River, NY, lot No. J5875.

[4]Section of Immunology and Cell Biology (Drs. Lichtenfeld, Desner, and Mardiney) and Section of Medical Oncology (Dr. Wiernik), Baltimore Cancer Research Center (BCRC), Division of Cancer Treatment (DCT), National Cancer Institute (NCI), Md.

[5]*Present address:* Long Island Jewish Medical Center, New Hyde Park, NY.

**Reprint requests to:* J. Leonard Lichtenfeld, MD, BCRC, DCT, NCI, University of Maryland Hospital, 22 S Greene St, Baltimore, Md 21201.

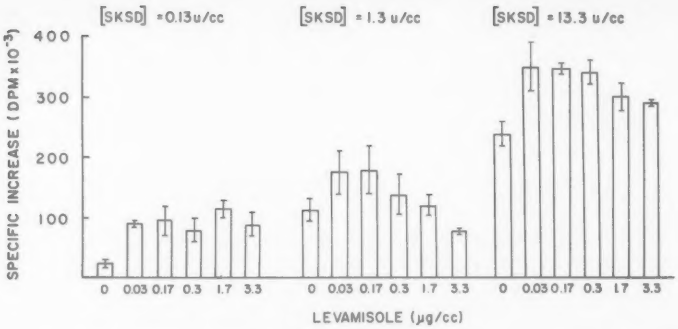

FIGURE 1.—Effect of levamisole on lymphocyte response to SKSD. Significant amplification with levamisole was observed with several concentrations of levamisole and SKSD (0.13 and 13.3 units/ml), but did not occur with 1.3 units/ml of SKSD at any concentration of levamisole (*see* text). ^{3}H-thymidine incorporation was measured on Day 7.

(DPM) for triplicate cultures ± the standard error. Any two means greater than three average standard errors apart are significantly different at greater than the 95% confidence level. The data were further evaluated by determining the specific increase (DPM of experimental cultures − DPM of control cultures). The reasoning behind this mode of data presentation has been discussed in detail elsewhere (11).

Preparation of levamisole, antigen, and PHA-M.— Levamisole was dissolved in RPMI 1640 media and utilized at concentrations specified in the text. SKSD (Varidase; Lederle Laboratories, Pearl River, NY) was prepared and utilized as previously reported (11). PHA-M (Difco, Inc, Detroit, Mich) was dissolved in 5 ml of RPMI 1640 media and was then further diluted (1:20) to obtain a 1:100 PHA-M stock solution. All concentrations described in the text and figures represent the final in vitro concentrations.

RESULTS

Levamisole improved the response of sensitized lymphocytes to specific antigen when multiple combinations of drug and antigen concentrations were evaluated simultaneously. Two representative experiments are shown in figures 1 and 2. In the first experiment (fig 1), levamisole increased thymidine incorporation at all drug concentrations compared to control cultures without levamisole in the presence of a suboptimal concentration of SKSD (0.13 units/ml). Levamisole also produced statistically significant increases in lymphocyte response in combination with an optimal stimulating concentration of SKSD (13.3 units/ml) and all drug concentrations except 1.7 μg/ml. In contrast, no concentration of levamisole produced significant amplification of response with 1.3 units/ml SKSD which is also a suboptimal antigen concentration.

A similar experiment is shown in figure 2. The addition of 0.03 and 0.17 μg/ml levamisole to cultures containing 1.3 units/ml SKSD resulted in enhanced response to the antigen, while no effect was found with 0.3 μg/ml. The opposite effect occurred at 13 units/ml SKSD, with amplification occurring only with 0.3 μg/ml of levamisole.

Levamisole also significantly improved lymphocyte response when the cells were stimulated by the mitogen PHA-M (fig 3). All concentrations of levamisole in this experiment amplified response with the suboptimal dilution (3000^{-1}) of the mitogen, while a similar effect was seen only with larger concentrations of levamisole (1.7, 3.3, and 33 μg/ml) when the optimal dilution of PHA-M (300^{-1}) was used.

Figure 4 illustrates the effect of levamisole on the one-way mixed leukocyte culture with increased incorporation of tritiated thymidine by lymphocytes in the presence of levamisole. A significant elevation of the specific increase occurred with the 3.33-μg/ml concentration of the drug. There was a slight increase in background counts in this experiment

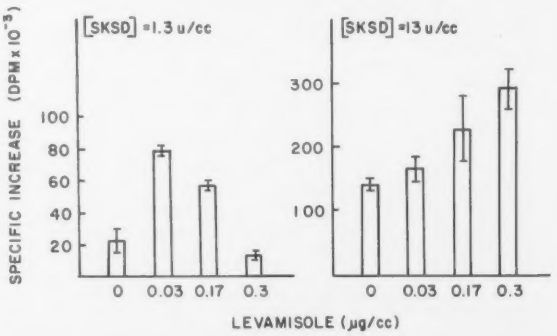

FIGURE 2.—Effect of levamisole on antigen response. Levamisole (0.03 and 0.17 μg/ml) amplified response to 1.3 units/ml of SKSD, while a similar effect was observed only with 0.3 μg/ml of levamisole at the 13-units/ml SKSD concentration.

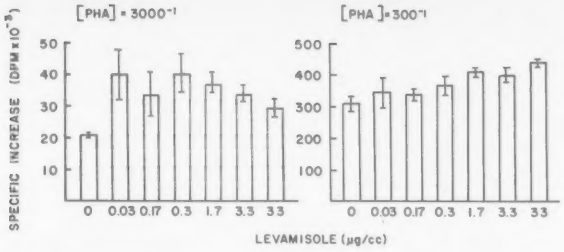

FIGURE 3.—Effect of levamisole on lymphocyte response to the mitogen PHA-M. Amplification of response occurred with multiple levamisole concentrations and suboptimal (3000^{-1}) and optimal (300^{-1}) stimulating dilutions of PHA-M (*see* text). ^{3}H-thymidine incorporation was determined on Day 4 of the culture.

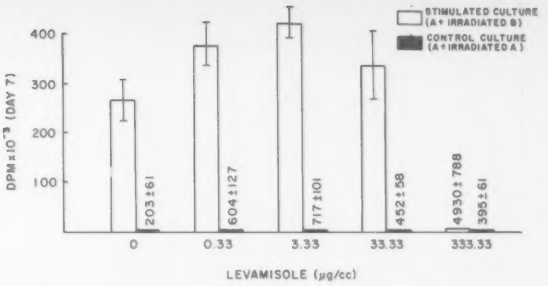

FIGURE 4.—Effect of levamisole in increasing concentrations on the 1-way mixed leukocyte reaction. The concentration of radiated stimulator and nonradiated responding cells was 1.5 × 10^{6} cells/culture. ^{3}H-thymidine incorporation was measured on Day 7.

when levamisole was added to cultures containing only cells, suggesting minimal effect of the drug itself on lymphocyte response.

The addition of large concentrations (333 μg/ml) of levamisole to cultures uniformly resulted in a marked decrease in the response of stimulated cells (fig 4). No viable intact cells were present in cultures containing this high concentration of levamisole when cells were stained with trypan blue, reflecting a direct cytotoxic effect of the drug. Cells cultured with drug concentrations of 33 μg/ml or less uniformly contained >90% viable lymphocytes on the 7th day of culture.

DISCUSSION

The results of these experiments demonstrate that levamisole has the capacity to modulate the response of lymphocytes stimulated in vitro with specific antigen, mitogen, and allogeneic cells and suggest a direct effect of the drug on the cell-associated immune system. Our data also indicate that detection of amplification was related to finding the unique combination of stimulus and levamisole concentrations for each individual culture which produced the augmented response. By culturing cells in a simultaneous panel comparing multiple concentrations of stimulus and levamisole, we have found significant increments in thymidine incorporation with suboptimal, supraoptimal, and optimal concentrations of the stimulus and with levamisole concentrations ranging from 0.017 to 33 μg/ml.

There have been conflicting reports of the in vitro activity of levamisole in human experimental systems. Copeland et al (12) found no evidence to suggest that levamisole improved in vitro lymphocyte response to single concentrations of stimulating cells and mitogen. Our experience was similar when limited combinations of stimulus and high concentrations of levamisole were evaluated. Other investigators (13,14) have also reported augmented response with levamisole added to stimulated lymphocyte cultures, although the concentrations (50–250 μg) required to produce this effect in one report (14) were considerably greater than the effective range found in our experiments. Levamisole alone had little effect on cells cultured without added stimulus, which is in agreement with the observations of other investigators (13,14). In murine systems however, levamisole alone has definite mitogenic properties (15,16).

The variable effects of levamisole on lymphocytes in vitro may reflect other factors associated with the culture system itself. We have reported elsewhere (17) the results of preliminary experiments which suggested that time manipulations relative to culturing technique may have an effect on producing improved response. The stability of levamisole under conditions of the culture system has not been explored. Although the drug remains stable in dry powdered form when stored up to 44 months,[6] degradation to inactive metabolites occurs at 37°C and at a pH level >4.0 (18). We are not aware of any stability studies on levamisole dissolved in RPMI 1640 or similar buffered balanced salt solutions. The probable instability of levamisole under these conditions may contribute to the inconsistent results reported from different laboratories.

A chemically defined immune-modulating agent such as levamisole represents a potentially valuable

[6]Stability report No. 549. Janssen Pharmaceutica, Beerse, Belgium.

addition to the treatment programs for cancer patients. However, the conflicting observations regarding the in vivo and in vitro activities of levamisole require further careful investigation to increase our understanding of this interesting immune-modulating agent.

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Trial of Heparin and Cyclophosphamide (NSC-26271) in the Treatment of Lung Cancer[1,2,3]

Herbert E. Edlis,* Arnoldus Goudsmit, Clyde Brindley, and Julian Niemetz[4,5]

SUMMARY

Preliminary reports have suggested that heparin may enhance the effectiveness of chemotherapy for lung cancer. The VALCSG initiated a study to evaluate further the efficacy and feasibility of administering a heparin and chemotherapy regimen. Treatment consisted of continuous iv heparin infusion for 48–72 hours followed by iv administration of cyclophosphamide (1100 mg/m^2) and continued treatment with heparin and warfarin. Only one of the 19 patients (5%) with nonresectable or metastatic lung cancer showed evidence of objective tumor response. This indicated no benefit from the addition of heparin. The study also revealed some of the limitations and problems of such a treatment program.

[Cancer Treat Rep 60:575–578, 1976]

To date, the treatment of nonresectable or metastatic lung cancer with cytotoxic agents has been generally unsatisfactory. Recent studies have suggested that a new approach in which cytotoxic agents are combined with heparin may be more effective. Elias and Brugarolas (1) noted tumor regressions in four consecutive patients with bronchogenic carcinoma who received one course of heparin followed by combination chemotherapy consisting of cyclophosphamide, 5-fluorouracil, 6-thioguanine, methotrexate, and vincristine. Two of these patients had previously failed to respond to the same chemotherapy given alone. A subsequent report described tumor regressions in nine of 12 patients treated with a similar heparin and chemotherapy regimen, while no responses were seen in 13 patients receiving chemotherapy alone (2,3). These findings suggested that heparin might enhance the cytotoxic effect of chemotherapy.

Interest in the use of anticoagulants in the treatment of cancer has been based primarily on studies indicating that fibrin deposition may be an important factor in the establishment and growth of metastases and perhaps in the growth of primary lesions as well (4–9). Also, there has been speculation that fibrin deposition by a growing tumor may shield it from cytotoxic drugs (4). Consequently, the suppression of fibrin formation by a drug such as heparin might inhibit tumor growth and/or increase its susceptibility to cytotoxic therapy. It should be noted, however, that the possible antineoplastic effect of heparin may be related to properties other than anticoagulant activity (10).

The VALCSG initiated a preliminary study to evaluate further the efficacy of heparin and chemotherapy for lung cancer and to determine the feasibility of administering such treatment in contemplation of a larger controlled cooperative study.

MATERIALS AND METHODS

Patients with nonresectable or metastatic lung cancer who had measurable lesions and a Karnofsky performance status >30 were considered for the study. Because of potential bleeding problems, patients with the following conditions were excluded: active peptic ulcer, gastrointestinal bleeding, hemoptysis, brain metastases, or hemorrhagic diathesis indicated by laboratory tests or history. The 19 patients completing treatment were all men with a median age of 55 years. Cell types consisted of

[1]Received Oct 2, 1975; revised Feb 26, 1976; accepted Mar 10, 1976.

[2]Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

[3]This study was conducted by the Veterans' Administration (VA) Lung Cancer Study Group (VALCSG).

[4]VA Hospitals: Bronx, NY (Drs. Edlis and Niemetz); Minneapolis, Minn (Dr. Goudsmit); and Mountain Home, Tenn (Dr. Brindley).

[5]We wish to acknowledge the technical assistance of Mr. Kenworth Jones and Mr. Manuel Reyes.

*_Reprint requests to:_ Herbert E. Edlis, MD, VA Hospital, 130 W Kingsbridge Rd, Bronx, NY 10468.

TABLE 1.—Characteristics of study group

Patient No.	Age (yrs)	Cell type	Extent of disease*	Measurable lesions	Fibrinogen level (mg/100 ml)	Performance status	Prior therapy
1	55	Undifferentiated large cell	E	Lung, node	300	80	None
2	50	Epidermoid	E	Node	460	70	None
3	50	Epidermoid	L	Lung	365	40	None
4	47	Epidermoid	L	Lung	515	80	None
5	46	Epidermoid	L	Lung	430	80	Procarbazine
6	81	Undifferentiated small cell	L	Lung	515	70	None
7	44	Epidermoid	E	Lung, node	275	40	Radiotherapy
8	59	Epidermoid	L	Lung	280	50	None
9	56	Undifferentiated large cell	E	Lung, liver	520	70	None
10	81	Adenocarcinoma	L	Lung	450	70	None
11	61	Adenocarcinoma	E	Lung, liver	—	40	None
12	66	Undifferentiated large cell	L	Lung	—	30	None
13	54	Undifferentiated small cell	E	Lung, node	—	70	None
14	50	Undifferentiated large cell	E	Lung, liver	—	30	None
15	65	Undifferentiated small cell	E	Lung, node	—	60	None
16	54	Unclassified	E	Lung, node	—	70	None
17	55	Epidermoid	E	Lung, liver, node	—	70	None
18	62	Epidermoid	E	Lung, node	—	60	None
19	53	Adenocarcinoma	E	Lung, liver, node	—	60	None

*E = extensive; L = limited.

epidermoid carcinoma (eight patients), adenocarcinoma (three), undifferentiated small cell carcinoma (three), undifferentiated large cell carcinoma (four), and unclassified (one). Extent of disease was limited to one hemithorax in seven patients while the remaining 12 had evidence of remote metastases. Karnofsky performance status was favorable (60–80) in 13 patients and relatively poor (30–50) in six. Six patients had fibrinogen levels >400 mg/100 ml. Seventeen patients had received no prior therapy. One patient had previously received radiotherapy and another had previously received procarbazine. Characteristics of the study group are listed in table 1.

Before initiation of therapy patients were screened with the following coagulation tests: bleeding time (Ivy method), clotting time at 37°C, celite cephalin time (activated partial thromboplastin time [PTT]) (11–13), fibrinogen and thrombin time (13), one-stage prothrombin test, and platelet counts with phase microscopy.

Heparin[6] was given as follows: 150 units/kg by iv push followed by continuous iv infusion of 600 units/kg/24 hours. Doses were increased progressively until a clotting time of three times the baseline level and an activated PTT of 100 seconds were reached. In most cases the clotting time was well over 20 minutes and the PTT was >2 minutes (control = 45–50 seconds). This level of heparinization was maintained for 48–72 hours at which time cyclophosphamide (1100 mg/m^2) was given iv and the heparin was continued for an additional 24 hours. In one patient heparin was given for 48 hours and in another for 72 hours after cyclophosphamide administration. In order to prevent "rebound hypercoagulability" and potential thromboembolic episodes, a short course of warfarin[7] was administered (Elias EG, personal communication). On the day cyclophosphamide was given, patients received warfarin (10 mg) followed by 5 mg for 2 days and 2.5 mg for 2 days.

Of the 27 patients entered in the study 19 completed at least one course of treatment. Of these, 11 patients received a second course and two received three courses at 3-week intervals.

Patients were followed with daily clotting and PTTs, blood counts as needed, and periodic assessments of measurable lesions and performance sta-

[6]Sodium heparin (beef lung) was obtained from The Upjohn Co, Kalamazoo, Mich; sodium heparin (porcine intestine) was obtained from Eli Lilly and Co, Indianapolis, Ind.

[7]Sodium warfarin (Coumadin) was obtained from Endo Laboratories Inc, Garden City, NY.

TABLE 2.—Treatment and results

Patient No.	No. of treatment courses	Tumor response*	Survival after treatment (wks)	Status
1	2	NR	60	Died
2	2	NR	31	Died
3	1	NR	5	Died
4	2	PR	14	Died
5	2	NR	84+	Alive
6	2	NR	38	Lost to followup
7	1	NR	5	Died
8	1	NR	4	Died
9	2	NR	26	Died
10	2	NR	16	Died
11	2	NR	12	Died
12	1	NR	2	Died
13	2	NR	17	Died
14	2	NR	17	Died
15	1	NR	4	Died
16	1	NR	42	Died
17	3	NR	16	Died
18	2	NR	12	Died
19	3	NR	18	Died

*NR = no response; PR = partial response.

tus. Tumor response was defined as at least a 50% reduction in one diameter of a measurable lesion. Informed consent was obtained from all patients entered in the study.

RESULTS

Only one of 19 patients (5%) completing treatment showed evidence of objective tumor regression. This was a partial response of 4 weeks' duration occurring 18 days after initiation of treatment. The other patients showed no signs of objective response and went on to develop progressive disease. The median survival after treatment was 16 weeks with a range of 2–84+ weeks. At present, 17 patients have died, one patient is alive at 84 weeks, and another was lost to followup 38 weeks after treatment (table 2).

In eight patients treatment could not be completed for the following reasons: (*a*) severe bleeding in two patients requiring discontinuation of heparin; (*b*) allergy to heparin in one patient; and (*c*) inability to maintain stable hypocoagulation during the required time in five patients.

Toxicity included four episodes of bleeding, two of which were severe. One patient developed hemoptysis which subsided upon discontinuation of heparin but another exsanguinated from massive gastrointestinal bleeding with autopsy evidence of hemorrhage from a small bowel metastasis. Two patients developed minimal gum bleeding but were able to complete treatment. Gastrointestinal and hematologic toxicity was minimal. Twelve patients developed mild to moderate vomiting and 14 patients became mildly leukopenic (3000–4000 leukocytes/mm^3). Toxicity is summarized in table 3.

TABLE 3.—Treatment-related toxic effects

Effects	Patients with toxicity			
	Mild	Moderate	Severe	Life-threatening
Vomiting	8	4	0	0
Bleeding	2	0	1	1
Leukopenia	14	0	0	0

DISCUSSION

The objective tumor response rate of 5% in this study indicated no benefit from the addition of heparin. It was perhaps even somewhat poorer than might have been expected with cyclophosphamide alone.

In comparing the lack of benefit in this study with the earlier reports (1–3) several factors may be considered. Cyclophosphamide was chosen rather than the five-drug regimen because it was assumed that the critical factor was the therapy with heparin. Furthermore, cyclophosphamide had been shown to prolong survival in patients with lung cancer (14) and has been used as the "control" regimen in VALCSG protocols for several years.[5] With regard to the anticoagulation regimen, it is not known whether intermittent or continuous treatment is preferable or whether the anticoagulation therapy should be heparin or warfarin; the earlier studies are not clear on these points. Also, the patients studied may not have been entirely comparable. Several of the patients in this study exhibited normal or only mildly elevated fibrinogen levels compared with the higher values noted in the patients studied earlier (1). Also, it is unclear whether the distribution of cell types is similar since this was not indicated in the earlier reports (2,3). Further study would be needed to conclusively resolve these questions.

Several problems were encountered in this study which should be considered in designing future trials. Despite the exclusion of patients at risk, serious hemorrhage occurred, particularly from tumor sites. Drug-induced thrombocytopenia might be expected to further aggravate this complication. Another problem was that a substantial number of patients, five of 27 (19%), were unable to complete treatment because of difficulties in achieving or

[5]VALCSG protocols 10–14, 1968–1974.

maintaining heparin effect despite repeated attempts. It is possible that tumor and/or host factors may have been responsible for this since in the other patients hypocoagulability was easily achieved. Also, attempting to achieve even and continuous heparin infusion rates required meticulous care and still was not always possible. In planning further studies on the use of heparin and chemotherapy for cancer it should be recognized that only selected patients are suitable and special medical, nursing, and laboratory support is required.

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Study of In Vivo Tumor State and Response to Therapy by Analysis of DNA Distribution[1,2]

Yosh Maruyama,[3,*] M. R. Raju,[4] Elvira Bains,[4] and James Jett[4,5]

SUMMARY

The growth characteristics of an in vivo tumor model were studied. This tumor offers an intermediate system useful for the study of tumor cells in the intact animal, but does not have the complexities of solid tumors. DNA distribution was correlated with macromolecular DNA synthesis. Mechlorethamine, BCNU, radiation, and hydroxyurea were studied and produced perturbation in stationary phase-cell kinetics. There was a redistribution of cell ages consistent with induced cycling activity with most, but not all, of the above agents. This indicated a recruitment from a stationary phase by cancer therapeutic agents, although subsequent cell-cycle traverse may also be greatly perturbed. A number of considerations important to the interpretation of this type of data are discussed.

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A cell in culture may be conveniently, easily, and uniformly manipulated and its responses accurately studied. However, a live animal tumor can follow an extremely wide variety of growth modes as it progresses in vivo. Hence, it is desirable to develop a number of well-characterized model tumor systems whose behavior is well understood for the evaluation of in vivo tumor response to therapeutic agents. Tumor systems particularly well studied for growth characteristics are the ascites tumors (1–3), eg, the LSA tumor, a lymphoblastic lymphoma syngeneic for the C57BL mouse. We have studied the characteristics of this tumor with regard to growth and response to therapy using several agents with different mechanisms of action. We have studied mechlorethamine, ^{60}Co gamma radiation, hydroxyurea, and BCNU.[6] Mechlorethamine and BCNU are alkylating agents and ^{60}Co gamma rays deliver ionizing radiation that is cycle nonspecific. Hydroxyurea represents an antimetabolite for DNA synthesis and is a cycle-specific agent (4). The effects of these agents on this model tumor system are described in this report.

MATERIALS AND METHODS

The LSA lymphoma of C57BL mice was adapted to ascites growth from a thymic lymphoma induced by the radiation leukemic virus agent (3). The ascites tumor was maintained by serial isologous ip passage into normal female mice at weekly intervals. The cells were harvested with ice cold (0°C–4°C) Hanks' balanced salt solution and transplanted at weekly intervals in a volume of 0.2 ml.

The tumor-growth curves were constructed after the ip inoculation of cells. At various intervals thereafter, a quantitative wash of the peritoneal cavity was done using heparinized normal saline (5). The cell number was determined from diluted samples counted with phase-contrast microscopy using a standard hemacytometer. Animals were randomly

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[3]Department of Radiation Medicine, Medical Center, University of Kentucky, Lexington.

[4]BioMedical Instrumentation Laboratory, Los Alamos, NM.

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*Reprint requests to: Yosh Maruyama, MD, Department of Radiation Medicine, Medical Center, University of Kentucky, Lexington, Ky 40506.

[6]Mechlorethamine: NSC-762; CAS reg. No. 55–86–7; diethylamine, 2,2'-dichloro-*N*-methyl-, hydrochloride.

Hydroxyurea: NSC-32065; CAS reg. No. 127–07–1.

BCNU: NSC-409962; CAS reg. No. 154–93–8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-.

selected for count after the injection of 1×10^6 tumor cells.

The tumor cells were harvested and suspended in 20% formol saline (Puck's saline solution) for 24 hours at 0°C–4°C. They were then suspended in 10% formol saline for subsequent Acriflavine-Feulgen fluorescent staining (6) and flow microfluorometric (FMF) study. DNA measurements were performed using the Los Alamos FMF system (7,8) and the number of cells was $1–2 \times 10^7$ per sample. Virtually identical results were obtained in separate studies performed with propidium iodine.

Tumors were exposed in vivo to ^{60}Co radiation at a whole-body dose of 1000 rads. Hydroxyurea (Squibb and Sons, Princeton, NJ) was given in a dose of 100 mg/mouse suspended in normal saline and injected ip (5 g/kg); mechlorethamine (Merck Sharp and Dohme, West Point, NY) was given in a dose of 0.025 mg/mouse ip (1.25 mg/kg); BCNU (Cancer Therapy Evaluation Program, Division of Cancer Treatment, NCI, Bethesda, Md) was given in a dose of 0.25 mg/mouse (12.5 mg/kg) ip or iv to compare the relative effect on tumor response of rapid and slow mixing, the latter through the normal circulatory mechanisms. All tumor manipulations were performed on Day 5 after the ip inoculation of 1×10^6 cells on Day 0. Animals injected with this cell dose will ordinarily die on approximately Day 9. Tumor-cell samples were obtained beginning 24 hours after the stated therapeutic exposure beginning on Day 6 and daily thereafter at 24-hour intervals to Day 8.

The LSA lymphoma shows a TD50 of one to two cells (the tumor-cell dose required to produce tumor takes in half of the normal recipients) and therefore most cells are clonogenic (9). This virulence was advantageous in this research since the responses observed represented the response of a clonogenic tumor stem cell. Cell lysis after irradiation is also rapid (unpublished data).

Tritiated thymidine (^{3}H-TdR) labeling studies were done in mice initially irradiated with 400 rads and then given tumor transplants (5). ^{3}H-TdR was obtained from Schwarz/Mann, Orangeburg, NY, and injected ip at a dose of 0.16 millicuries/g. The uptake period was 30 minutes and was terminated by the injection of 10 μmols of unlabeled thymidine. The tumor was harvested, washed with cold perchloric acid (PCA) and alcohol-ether, and solubilized with tissue solubilizer. An aliquot was counted in a liquid scintillation counter to determine the uptake per cell.

Analysis of FMF Spectra

The mathematic analysis of DNA distributions derived from FMF and the measurement of DNA content of single cells are readily accomplished using the method described by Dean and Jett (10). Briefly, three subpopulations are assumed to exist (ie, 2C or G_1, 4C, or G_2-M) and an intermediate state of DNA content corresponding to S phase. The G_1 and G_2-M subpopulations would be expected to fall into single channels of the pulse-height analyzer separated by a factor of 2. Dean and Jett (10) analyzed such data using a least squares best fit of a normal distribution to the G_1 and the G_2-M peaks. They attributed the shape of the cell distribution to electronic noise, light scattering, and nonuniform staining procedures. Computer analysis has been shown to yield data in close agreement with autoradiography, comparing curve analysis and labeling index (10,11) and fitting a second-degree polynomial to the intermediate S-phase distribution.

RESULTS

Tumor Growth and ^{3}H-TdR Labeling

A 30-minute pulse of ^{3}H-TdR was given to tumor cells on different days of growth. The uptake was

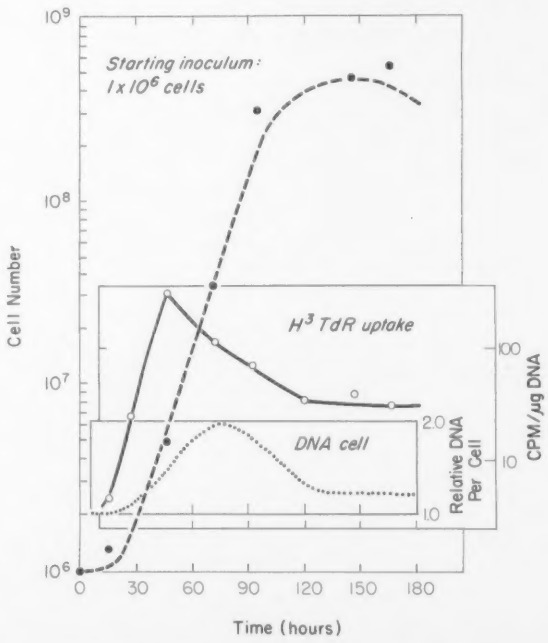

FIGURE 1.—Growth curve for LSA tumor. Early stationary phase attained in approximately 5 days. Insert figures trace ^{3}H-TdR uptake and DNA/cell changes (by diphenylamine reaction; *see* ref 5) with growth stage. Diploid DNA content of cells doubles during DNA-S to become tetraploid and divides with mitosis to return to diploid. DNA/cell changes proceed concurrently with ^{3}H-TdR labeling changes.

terminated by the injection of 10 μmols of unlabeled thymidine. The results of such an experiment starting with an inoculum of 1×10^6 cells are shown in figure 1. The DNA-synthetic activity preceded and rose concurrently with the cell-number increase. Peak activity was attained on Day 2 or during early tumor growth. During the mid-exponential phase (45–90 hrs) a slowing began, and during the stationary phase DNA-synthetic activity declined. During the mid-growth cycle (~ 100 hrs) DNA synthesis was still active but was minimal in the aged tumor (5,12), indicative of reduced proliferative activity.

DNA Distribution During Tumor Growth

The Acriflavine-Feulgen stain (13) can be measured using fluorescence response with dye that has reacted with cell DNA using an FMF device. A typical FMF curve (fig 2) shows the distribution of DNA contents of cells with a bimodal distribution (normal spleen cells). The left peak represents G_1 cells with a diploid DNA (2C) content. The right peak represents G_2-M cells with a tetraploid DNA (4C) content. The intermediate peak represents cells in DNA synthesis (S) with widely varying DNA contents. Dean and Jett (10) have developed a computer program which can partition cells into G_1, G_2-M, and S based upon best fits and curve fitting.

Figure 2 shows data for the LSA ascites tumor growing in the intact animal (6). Figure 3 shows DNA-distribution data from a typical tumor-growth experiment. The transplant ascites lymphoma shows an aneuploid DNA cell content. There was a large peak from samples obtained on Days 1 and 2 with a DNA content nearly the same as the normal spleen cells. The cells of this peak were separated in the cell separator and were found by microscopic examination to be normal white cells.

During early growth, DNA S-phase cells represented a large fraction of the population. After 120 hours a tumor-cell population had accumulated and most of the cells were in G_1 phase and remained there beyond 168 hours. The recipients began to die of disseminated tumor after 8–9 days (200 hrs). Thus, the tumor collected in the G_1 phase as the host animal approached a preterminal or terminal phase (5,6). Relatively few tumor cells were in the G_2-M phase.

Study of Kinetics and Perturbation of Advanced Tumor by Cancer Therapeutic Agents

The agents studied in these experiments on the LSA tumor were ^{60}Co gamma radiation, the alkylating agents mechlorethamine and BCNU, and the antimetabolite hydroxyurea.

The tumor was exposed on Day 5 of tumor growth at which time it was in an early stationary phase. Samples were obtained beginning on Day 6 and daily thereafter. FMF-distribution curves are shown in figures 3 and 4. Figure 3 shows the age-distribution curves for the undisturbed tumor growing in the intact mouse. On Day 5 cycling S and G_2-M cells were evident and decreased on subsequent days. Table 1 shows data obtained for ^{60}Co gamma radiation, mechlorethamine, BCNU, and hydroxyurea.

Mechlorethamine

After mechlorethamine exposure on Day 5, tumor samples obtained on Days 6 and 7 showed extensive debris. However, little return of the spectra to favor large numbers of S-phase cells was detected afterwards (table 1). The FMF DNA-distribution curves did not show redistribution to a significant number of S or G_2-M cells. Other experiments have indicated

TABLE 1.—Tumor cell-DNA distribution after therapy
(percent of population)

	% of population in indicated cell-cycle phase on Day—											
	5			6 (24 hrs after treatment)			7 (48 hrs after treatment)			8 (72 hrs after treatment)		
Treatment	G_1	S	G_2-M	G_1	S	G_2-M	G_1	S	G_2-M	G_1	S	G_2-M
None	49.5	41	10	64	28	8	68	21	11			
Mechlorethamine	Treatment day				—			—		73	18	9
Radiation (1000 rads)	Treatment day			47	26	26	56	32	13	58	19	23
Hydroxyurea	Treatment day			39	53	8	48	43	10	49	38	13
BCNU,* ip	Treatment day			33	40	27	50.5	40	10	35	30	35
BCNU, iv	Treatment day			58	27	14.5	47	41	11.5	31.5	11	58

*See fig 4 for representative curves.

a greater sensitivity of the tumor to cycle-specific agents 1 day after mechlorethamine treatment (12).

^{60}Co gamma radiation

Table 1 shows the sequential changes in age distribution of tumor cells observed after 1000 rads of whole-body exposure of tumor-bearing animals. The tumor obtained on Day 6 shows an increase in G_2-M cells consistent with a radiation-induced G_2 block. On Days 6–8 there was a larger cohort of S and G_2-M cells which progressively declined. This indicates an induced recycling activity caused by the radiation.

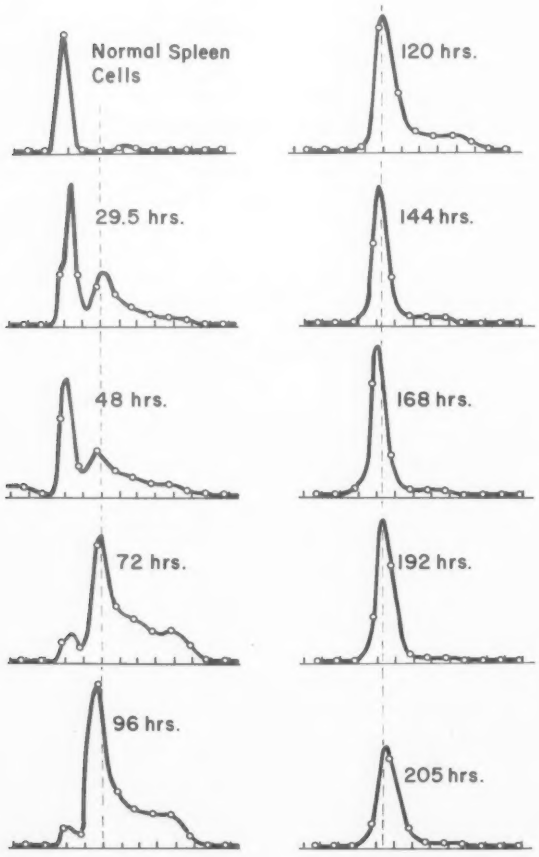

FIGURE 2.—Serial FMF spectra with tumor growth and progression. Upper left figure represents normal spleen cells. Sample at 29.5 hrs has shown a peak representing normal cells (separated and identified on cell sorter) of the peritoneal cavity. The tumor cells gradually replace and overgrow normal background cells. Advanced tumor is largely arrested in G_1 phase.

Hydroxyurea

Table 1 shows the sequential changes in age distribution of tumor cells observed after exposure of the tumor to 100 mg/mouse of hydroxyurea on Day 5. There was an increased fraction of cells in S phase consistent with stimulated cycling activity of the LSA tumor; this fraction declined progressively over Days 6–8. This has also been reported in other systems.

BCNU given ip and iv

Table 1 and figure 4 show results observed when BCNU was injected ip. BCNU represents a very potent antitumor agent against the LSA tumor. There was stimulation and a shift in the age distribution of the tumor to favor a larger number of S-phase cells. On Day 8 a delayed G_2 accumulation of cells was evident.

Table 1 shows results observed when BCNU was injected iv on Day 5. A G_2 accumulation of tumor cells was again observed although the maximum effects were again delayed in appearance. A larger cohort of G_2-M–phase cells was observed up to the third day after exposure of tumor to BCNU. It appears that a persistent and delayed G_2 blockade remained in the cells surviving the BCNU exposure (14–17).

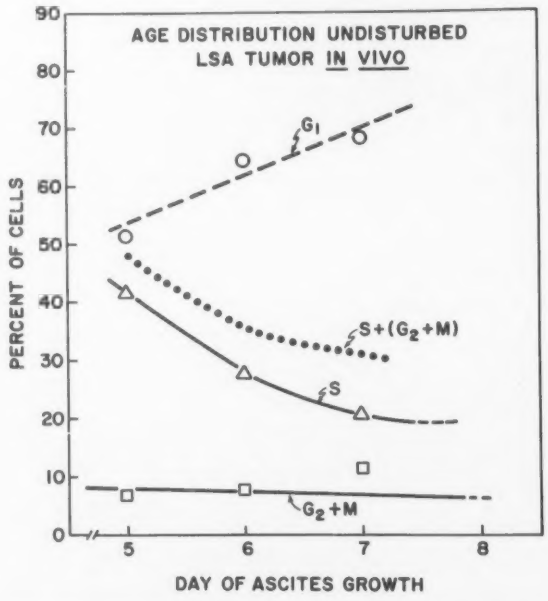

FIGURE 3.—Tumor cell-age distribution with tumor growth during Days 5–8. Total fraction of cells in S, S + (G_2-M), and G_2-M are shown.

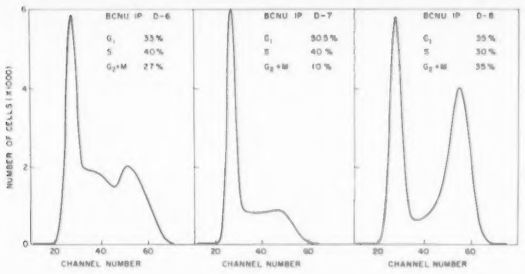

FIGURE 4.—Serial FMF spectra on Days 6–8 after ip BCNU exposure.

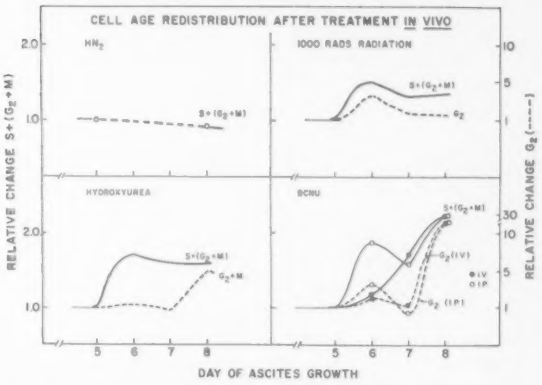

FIGURE 5.—Cell-age redistribution after treatment in vivo. Change is shown in relation to expected distribution found in undisturbed tumor growing in peritoneal cavity. Total fraction of cells in S + (G_2-M) and in G_2-M are shown.

Cell-Age Redistribution of Tumors After Treatment In Vivo

The cell-age distribution of the undisturbed tumor was used as a reference to determine the relative cell-age redistribution caused by the cancer therapeutic agent. The relative changes caused by mechlorethamine, ^{60}Co gamma radiation, hydroxyurea, and BCNU (iv and ip) are shown in figure 5. No change is shown by a horizontal line, and a shift to larger fractions of S or G_2-M cells is shown by a rising curve. A change in the DNA-distribution pattern was found, indicating a change in the cell-age distribution to contain "cycling" cells (position in cell cycle in S or G_2-M) after radiation, hydroxyurea, and BCNU (iv and ip). Mechlorethamine showed minimal activity which was difficult to interpret because of interfering cell and nuclear debris. Cell-cycle progression and traverse perturbation were also observed with cells blocked by BCNU in G_2-M phase.

DISCUSSION

Analysis of DNA distribution by methods such as FMF and its usefulness were studied in a model ascites tumor system in vivo. This method allows rapid and accurate quantitation of cell populations and cell-cycle parameters by analysis of cell distribution in different cell-cycle phases (15,18). To use the FMF method several conditions must be met: (*a*) clonogenic stem-cell response: tumor stem cells should be studied; (*b*) cell death: should be rapid to avoid populations with large proportions of dead cells; (*c*) viable or surviving viable cells should be studied after exposure to agent in order for a biologically important population to be studied; (*d*) time: cell sampling must be delayed to ensure that sufficient time has elapsed for cell response to begin and cell-age redistribution and reassortment to take place (dead cells could also disappear); (*e*) excessive cell debris should be avoided; and (*f*) subcurative doses should be used to leave an adequate number of viable cells.

With this information it is possible to determine the effects of cancer therapeutic agents on cell-kinetic behavior and to provide a means of tumor monitoring in vivo for designing effective, sequential, multi-agent, or multimodality therapy regimens. In the clinically apparent tumor, our studies show that the G_1-arrested noncycling cell represents an increasingly important therapeutic problem for the in vivo tumor (5,6,12). Whether this cell truly deserves the connotation of being in G_0 or a special resting state is probably unimportant, and, in fact, noncycling activity can no doubt occur in S, G_2, or even M phase. Quantitatively, however, G_1 cells represented the most numerous cell age present and a recycling activity which is induced must be effective against the G_1 cell-cycle stage.

In this tumor cells were collected in G_1 as the tumor became advanced and entered a nonproliferating state. All cells appeared capable of response to the agents tested by re-entering the cell cycle, although cell-cycle traverse may be greatly altered or inhibited by the perturbation produced by the therapeutic agent. The response to these agents or drugs depends in all likelihood on the extent of cell kill and lysis, the physiologic state of the host, the rate of clearance of toxic materials and cell debris, the restoration of a normal internal milieu, and the rate of response of surviving cells (some sublethally damaged) to re-enter the active cell cycle.

In this study we have tested several anticancer agents for their activity against the ascites tumor in early stationary phase in vivo. Based upon the results of these experiments, mechlorethamine was very weakly effective at a dose of 0.025 mg/mouse ip (2 mg/kg) in altering the age distribution to the tumor. ^{60}Co irradiation (1000 rads) induced cell-cycling activity with an early G_2 progression block. Hydroxyurea in a dose of 100 mg/mouse ip (5 g/kg) greatly altered age distribution and stimulated larger numbers of cells into S phase shortly after administration. BCNU, whether given ip or iv, stimulated cell redistribution into S and G_2-M phases, but a delayed G_2 block was apparent 3 days later (14–17). This phenomenon has been described by others. In experiments reported elsewhere (12), we have found that BCNU followed by 5-fluorouracil on a daily schedule was of much greater potency than either agent alone. Moreover, the combination of a chloronitrosourea followed by 5-fluorouracil led to long-term cures of the LSA tumor. This suggests that cell-kinetic data are relevant to planning therapy.

These factors also indicate that the method of DNA-distribution analysis by the FMF method is useful in describing cell-age distribution modifications produced by cancer chemotherapeutic agents or radiation therapy. After these exposures, the surviving population recovers from the effects of perturbation by the agent(s) by a redistribution of cells in a cycling population pattern and re-entry into a proliferating state. However, cell-cycle traverse could be greatly perturbed. We conclude that such information would be invaluable as a guide and monitor for therapy as well as in the selection of potential cancer agents which could be applied. The selection of an agent which can stimulate the nonproliferating to a proliferating compartment is most important. That transition is necessary for other antimetabolites to be maximally effective.

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Maintenance of Remission in Adult Acute Nonlymphoblastic Leukemia Using Intermittent Courses of Cytosine Arabinoside (NSC-63878) and 6-Thioguanine (NSC-752)[1,2]

James O. Armitage* and C. Patrick Burns[3,4]

SUMMARY

Twenty adults (median age, 58 yrs) with acute nonlymphoblastic leukemia who had achieved complete remission were given maintenance therapy with monthly courses of cytosine arabinoside and 6-thioguanine, each in a dose of 100 mg/m² every 12 hours for ten doses. The toxicity was minimal with no severe infections, bleeding, or treatment-related deaths. The treatment was often administered on an outpatient basis. The predicted life-table median remission duration is 14 months with 11 patients still in remission from 3+ to 29+ months.

[Cancer Treat Rep 60:585–589, 1976]

Several currently available remission-induction regimens for adult acute nonlymphoblastic leukemia (ANLL) have been shown to be successful in approximately one half of the patients who receive them. Unfortunately, the remissions tend to be short and many of the treatment programs used to maintain them are toxic or inconvenient to administer. We have used intermittent courses of cytosine arabinoside (ara-C) and 6-thioguanine to maintain remissions in adult patients with ANLL. The results, although preliminary, suggest that this combination is an effective and convenient regimen with relatively low toxicity.

PATIENTS AND METHODS

Twenty-six newly diagnosed adults with ANLL, who were seen at the University of Iowa Hospitals between January 1973 and July 1975, achieved complete remission. The diagnosis of ANLL was established by review of Wright's stained peripheral blood and bone marrow smears and appropriate cytochemical studies (1). Eighteen of these 26 patients were induced with ara-C and 6-thioguanine (2). Four patients received ara-C, 6-thioguanine, daunorubicin, vincristine, and prednisone (3), three patients received cyclophosphamide, vincristine, ara-C, and prednisone (COAP) (4), and one patient received daunorubicin and prednisone (5). Six patients did not receive intermittent ara-C and 6-thioguanine because of either their personal choice or a decision by their primary physician. Two of these patients received no maintenance therapy and four were maintained with other regimens (three patients received COAP and one patient received vincristine, methotrexate, BCNU, 6-thioguanine, cyclophosphamide, hydroxyurea, and daunorubicin) with remission durations varying from 1 to 6 months. The other 20 patients, who are discussed in this report, received 100 mg/m² of ara-C iv every 12 hours and 100 mg/m² of 6-thioguanine orally every 12 hours for 5 days at monthly intervals. Two patients initially received fewer than ten doses/course but were subsequently increased to the full dose. For some patients, the interval between courses was lengthened (maximum, 2 months) after the remission had been successfully maintained for several months. During remission, the patients were seen at least once a month and had a physical examination, laboratory evaluation, and bone marrow examination before each course of maintenance therapy. Several patients had the maintenance therapy administered by their local physicians, usually as an outpatient, but all patients were seen by

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[3]Division of Hematology-Oncology, Department of Medicine, University Hospitals, Iowa City.

[4]We wish to acknowledge the assistance of house officers and attending staff of the Department of Medicine (University Hospitals, Iowa City) who participated in the care of our patients.

*_Reprint requests to:_ James O. Armitage, MD, Department of Medicine, University Hospitals, Iowa City, Iowa 52242.

us before each maintenance course. The criteria for evaluation of response were those of the Acute Leukemia Group B (6). A relapse was diagnosed when the patient no longer had an M_1 marrow. Remission duration was defined as the time from first achieving an M_1 marrow to relapse. No patient had an extramedullary relapse at the time of a persistent M_1 marrow. Survival was calculated as the time from diagnosis to death. In each instance, the interval from diagnosis to initiation of treatment was <1 month. The data were reported as of November 1975 and were analyzed by the life-table method.

RESULTS

The age, sex, subtype of ANLL, remission-induction drugs, first remission duration, and survival of our 20 patients are presented in table 1. The median age was 58 years. The median duration of remission of the group has not yet been reached, but it is predicted by life-table analysis (fig 1) to be 14 months. Eleven of the 20 patients continue in complete remission from 3+ to 29+ months. No patient has had maintenance therapy stopped during continuing remission. A life-table analysis for the survival of this group of patients is also presented in figure 1. With 14 of the patients still alive (11 in first remission, one in second remission, and two in relapse) it is too early to accurately predict a median survival time.

The toxicity of this drug regimen has been minimal. In no instance was it necessary to reduce the amount of drugs given because of excessive myelosuppression. No patient has died or has had life-threatening infections or bleeding during remission. Three patients (Nos. 1, 11, and 12) had hepatocellular liver toxicity which probably resulted from treatment. In two patients this toxicity was manifested only as a transient rise in hepatic enzyme levels and in one additional patient as an enzyme elevation plus hyperbilirubinemia. Temporary interruption of therapy was necessary for only one patient (No. 12). Gastrointestinal toxicity has been minimal; when it occurred, it was generally controlled with anti-emetics. The therapy regimen has been well accepted by most patients, including those who received the drug from their local physicians. However, for one patient monthly therapy caused great emotional upset which led to a lengthening of the time between his courses and a relapse at 20 months.

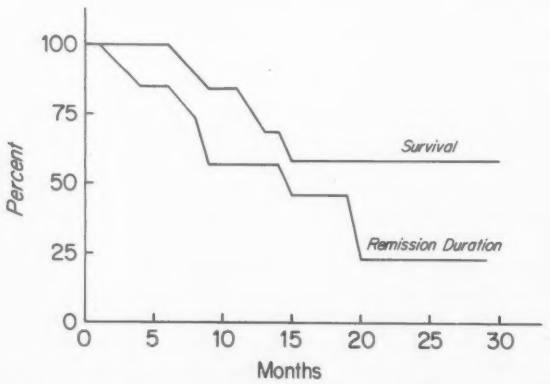

FIGURE 1.—Life-table analysis of remission duration and survival in patients with ANLL who received remission-maintenance therapy with ara-C and 6-thioguanine.

DISCUSSION

The remission durations reported for ANLL in adults with and without various forms of maintenance therapy are summarized in table 2. The median duration of unmaintained remission in adult ANLL is approximately 3 months (7,8). Many attempts to lengthen this discouraging figure have been made. These include oral antileukemic agents administered in a manner similar to that often employed for maintenance in acute lymphoblastic leukemia in children (9,10), intermittent re-administration of the induction drugs (4,11), cyclic regimens involving multiple drugs (12), and immunotherapy with or without concomitant chemotherapy (10,11). The data in table 2 suggest that some regimens result in only questionable improvement compared to no maintenance while others lead to seemingly definite prolongation of remission. The best results have been with therapy regimens that employ intermittent, intensive courses of antileukemic drugs. This contrasts with the findings in acute lymphoblastic leukemia in children where most of the programs currently used rely largely on continuous use of the most effective drugs. Several maintenance programs have shown median remission durations of 10 months or longer. These include the L-6 multiple-drug protocol (12), courses of COAP at 3-week intervals (4), monthly ara-C or 6-mercaptopurine and methyl-GAG (13), intermittent BCNU and cyclophosphamide (14), and our study of monthly ara-C and 6-thioguanine.

With several effective approaches available, the selection of a maintenance regimen will depend upon the relative ease of administration for both patient and physician, and the availability and relative toxicity of the drugs. The drugs we used, ara-C and 6-thioguanine, are readily available and familiar to all physicians involved in the management of acute leukemia in adults. When used in 5-day

TABLE 1.—Characteristics of adult patients with ANLL who had remission maintenance with intermittent ara-C and 6-thioguanine (TG)

Patient No.	Age (yrs)/sex	Subtype of ANLL*	Induction therapy regimen	Remission duration (mos)†	Survival (mos)‡
1	25/M	AML	ara-C+TG	20	26+
2	54/M	AML	ara-C+TG	18+	21+
3	63/M	AML	ara-C+TG	9	12
4	46/M	AML	ara-C+TG+daunorubicin + vincristine + prednisone	7+	10+
5	64/F	AML	ara-C+TG	5+	11+
6	70/F	AML	ara-C+TG	4+	6+
7	37/M	AML	ara-C+TG	3+	8+
8	60/M	AMMOL	ara-C+TG	29+	30+
9	70/M	AMMOL	ara-C+TG	18+	19+
10	17/F	AMMOL	ara-C+TG	15	21+
11	38/M	AMMOL	ara-C+TG	13+	17+
12	53/M	AMMOL	ara-C+TG	9	15
13	57/M	AMMOL	ara-C+TG	10+	13+
14	21/M	AMMOL	ara-C+TG+daunorubicin + vincristine + prednisone	8+	10+
15	59/M	AMMOL	ara-C+TG+daunorubicin + vincristine + prednisone	7+	9+
16	64/M	AMMOL	ara-C+TG	3	7
17	65/F	AMMOL	ara-C+TG	2	8
18	64/M	AMOL	ara-C+TG	7	13
19	67/F	AMOL	ara-C+TG	4	9
20	23/M	AEL	Daunorubicin + prednisone	8	12+

*AML = acute myeloblastic leukemia; AMMOL = acute myelomonoblastic leukemia; AMOL = acute monoblastic leukemia; AEL = acute erythroleukemia.
†From day of remission to relapse.
‡From diagnosis to death.

courses each month as described here, the drugs can be administered on an outpatient basis with minimal disruption of the patient's daily activities. Although advanced age has been documented as an adverse prognostic factor in acute leukemia (15), one study found no significant difference in the duration of complete remissions of older and younger patients (16). The relatively older age of our patients did not result in excessive toxicity or short remission durations. Although these results are preliminary, the ease of administration and paucity of side effects makes this an attractive regimen for remission maintenance in ANLL in adults. This regimen may be particularly applicable to older patients.

ADDENDUM

As of April 1, 1976, nine of the 20 patients remain in first remission with a life-table predicted median duration of 16 months.

LIST OF COMPOUNDS

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

COAP: cyclophosphamide, vincristine, cytosine arabinoside, and prednisone

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate

TABLE 2.—Duration of remission of ANLL in adults with or without maintenance therapy*

Ref No.	No. of patients	Median age (yrs)	Remission-maintenance regimen	Median duration of remission (mos)	Induction therapy regimen
7	17	50	None	2	Daunorubicin or 6-mercaptopurine + vincristine + methotrexate + prednisone
8	Pooled data	†	None	3	Variable
5	10	38	Hydroxyurea	6	Daunorubicin + prednisone
9	13	50	6-Mercaptopurine + methotrexate	6	Ara-C + daunorubicin
11	19	44	Monthly ara-C + TG alternating with ara-C + daunorubicin	6	Ara-C + daunorubicin
10	23	43	Methotrexate	6	Ara-C + TG or ara-C + TG + daunorubicin
10	18	40	Methotrexate + bacillus Calmette-Guérin	9	Ara-C + TG or ara-C + TG + daunorubicin
11	25	42	Monthly ara-C + TG alternating with ara-C + daunorubicin + immunotherapy‡	10	Ara-C + daunorubicin
12	46	45–50	L-6 multiple-drug therapy	10§	Ara-C + daunorubicin
4	17	50	COAP q3wks	10	COAP
13	16	<50	Monthly ara-C or 6-mercaptopurine + methyl-GAG	12§	Ara-C + daunorubicin
Present study	20	58	Monthly ara-C + TG	14§	Variable
14	13	37	BCNU + cyclophosphamide	15§	Cyclophosphamide + ara-C + vincristine

*Only studies with at least 10 adults are included.
†Not stated.
‡Allogeneic irradiated leukemic cells and bacillus Calmette-Guérin.
§Life-table analysis.

Cytosine arabinoside: NSC-63878; CAS reg. No. 69–74–9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride
Daunorubicin: NSC-82151; CAS reg. No. 23541–50–6
Hydroxyurea: NSC-32065; CAS reg. No. 127–07–1
6-Mercaptopurine: NSC-755; CAS reg. No. 6112–76–1; purine-6-thiol, monohydrate
Methotrexate: NSC-740; CAS reg. No. 59–05–2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-
Methyl-GAG: NSC-32946; CAS reg. No. 24968–67–0; guanidine, 1,1′-[(methylethanediylidene)dinitrilo]di-, dihydrochloride, monohydrate
Prednisone: NSC-10023; CAS reg. No. 53–03–2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-
6-Thioguanine: NSC-752; CAS reg. No. 1832–72–0; purine-6-thiol, 2-amino-
Vincristine: NSC-67574; CAS reg. No. 2068–78–2; leurocristine, sulfate (1:1)

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Study of Cytosine Arabinoside (NSC-63878) Synchronization Plus Vincristine (NSC-67574), Prednisone (NSC-10023), and L-Asparaginase (NSC-109229) for Remission Induction in Advanced Acute Leukemia in Children[1,2,3,4]

Wataru W. Sutow, David Thomas, C. Philip Steuber, Jeanette Pullen, Tribhawan Vats, J. Hugh Bryan, and S. K. Morgan[5,*]

SUMMARY

Cytosine arabinoside (CA) was utilized in efforts to synchronize leukemic cells in DNA synthesis for treatment with vincristine, prednisone, and L-asparaginase in children with acute leukemia in relapse. The results did not indicate any therapeutic advantage for patients treated with this combination compared to those treated without any attempt at CA synchronization.

[Cancer Treat Rep 60:591–594, 1976]

The results of previous studies by the SWOG have indicated that the combination of vincristine, L-asparaginase, and prednisone (VLP) induced remissions more frequently in advanced acute leukemia in children (76% of 46 evaluable patients) than L-asparaginase alone (54% of 28 patients) (1). In children presumably resistant to vincristine and prednisone (VP), the VLP regimen produced remissions more frequently (71% of 24 children) than L-asparaginase alone (59% of 17 children) (1). In another study, the administration of VLP in the same doses and schedule utilized in the present study produced remissions in 71% of 188 previously treated children with acute leukemia.[6]

Cytosine arabinoside (CA) used alone in varying dose schedules has induced CRs and PRs in 12%–33% of children with nonmyelocytic leukemia (2–4). The combination of CA and L-asparaginase has produced CRs in 15 (68%) of 22 children with advanced acute lymphocytic leukemia (ALL) (5). Eight were in remission after the 2nd week of treatment and seven after the 4th week. Using different schedules of CA, others have induced CRs and PRs in 13 (81%) of 17 and in 24 (32%) of 74 children with previously treated ALL (6,7).

Lampkin et al (8–11) have shown that CA injected rapidly iv at a dose of 5 mg/kg of body weight appears to synchronize leukemic cells in the S phase of the mitotic cycle. Utilizing the therapeutic strategy of synchronizing in the sensitive phase to produce cells most susceptible to the action of another mitotic-cycle-dependent drug (eg, vincristine), Lampkin et al were able to induce remissions in children with ALL (8,9). Vincristine was administered "when the greatest number of cells were thought to be in DNA synthesis" (9). Recruitment of

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[3]Cytosine arabinoside: CAS reg. No. 69–74–9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride.

Vincristine: CAS reg. No. 2068–78–2; leurocristine, sulfate (1:1).

Prednisone: CAS reg. No. 53–03–2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-.

L-Asparaginase: CAS reg. No. 9015–68–3.

[4]This study was conducted by the Southwest Oncology Group (SWOG) between Nov 20, 1973, and Oct 10, 1974 (SWOG protocol 7319).

[5]Department of Pediatrics, The University of Texas System Cancer Center, Houston (*Dr. Sutow*); Department of Biomathematics, University of Texas M. D. Anderson Hospital and Tumor Institute, Houston (*Dr. Thomas*); Department of Pediatrics, Baylor College of Medicine, Houston, Tex (*Dr. Steuber*); Department of Pediatrics, Children's Hospital, University of Mississippi Medical Center, Jackson (*Dr. Pullen*); Department of Pediatrics, University of Kansas Medical Center, Kansas City (*Dr. Vats*); Department of Pediatrics, University of North Carolina School of Medicine, Chapel Hill (*Dr. Bryan*); and Department of Pediatrics, Medical University of South Carolina, Charleston (*Dr. Morgan*).

**Reprint requests to:* SWOG, Operations Office, 3500 Rainbow Blvd, Suite 201, Kansas City, Kan 66103.

[6]SWOG. Unpublished data.

resting cells into the mitotic cycle has also been noted following CA synchronization with subsequent administration of vincristine (9).

The present study was intended to exploit the approach suggested by Lampkin et al (10,11) and to determine if an effort to synchronize leukemic cells in DNA synthesis would result clinically in increased frequency of remissions following treatment with a combination-drug regimen (VLP) known to be significantly effective. The period of 48 hours was selected as the optimum time for CA synchronization of leukemic cells in DNA synthesis (8).

METHODS

Children < 15 years of age were eligible for this study if they had a bone marrow diagnosis of acute leukemia (all morphologic types) in relapse and had had prior definitive therapy. Patients were considered eligible for this study even if the prior therapy included repeated courses of VP. Patients who had had previous therapy with no more than one course of L-asparaginase were also entered into the study if there was no definite history of resistance to L-asparaginase therapy. Those who had had prior treatment with CA and those who had become resistant to L-asparaginase were excluded from the study.

Eligible patients were randomly assigned to one of two treatment regimens: VLP or CALASP (cytosine arabinoside, vincristine, prednisone, and L-asparaginase).

VLP Regimen

Vincristine was given iv at a dose of 2 mg/m^2 weekly for five doses. Prednisone was started on the 1st day of treatment at a dose of 60 mg/m^2 daily (maximum of 60 mg/day) in three or four divided doses orally for 28 days. Prednisone was then discontinued in decremental fashion over the next 7 days. L-Asparaginase was administered iv at a dose of 500 IU/m^2 daily for 15 doses. If the bone marrow after 15 doses showed improvement to an M_2 but not to an M_1 status, seven additional doses of L-asparaginase were given.

CALASP Regimen

CA was administered at a dose of 150 mg/m^2 as a rapid iv infusion over a period of 3–5 minutes on the 1st day of treatment. Subsequent doses of CA were given on Days 7 and 14. If the bone marrow status on Day 17 had improved but was not in M_1 remission, an additional dose of CA was given on Day 21. The doses and schedules for the vincristine, prednisone, and L-asparaginase of the CALASP regimen were the same as those outlined above for the VLP regimen. However, the three drugs were started 48 hours after the administration of the initial pulse of CA.

Evaluation of Response

The patient's clinical course was monitored by regularly scheduled blood counts, physical examination, and biochemical tests. The latter included the determinations of amylase and fasting blood sugar levels, serum creatinine, blood urea nitrogen, uric acid levels, and liver isoenzymes. Urinalyses were also done regularly. Bone marrow examinations were conducted at the start of therapy and at the end of the 2nd and 3rd weeks of treatment. In a few cases, additional bone marrow examinations were done at the 4th and/or 5th week of treatment. The assessment of the bone marrow status was based on standardized criteria utilized in the SWOG studies of acute leukemia in children (12). The achievement of an M_1 or an M_2 status in the bone marrow was defined as a remission.

RESULTS

A total of 134 patients were considered eligible for the study. Among these, one did not receive L-asparaginase and four died of disease within the first 5 days of treatment. Data from the remaining 129 patients were utilized in these analyses. Sixty-five patients were randomized to the VLP treatment and 64 patients to the CALASP treatment. The major characteristics of the patients treated by the two regimens are outlined in table 1. The patients comprising each group appeared to be similar.

Data showing the response (achievement of M_1/M_2 status in bone marrow) to the two treatment regimens have been summarized in table 2. Remissions of bone marrow were achieved in 75% (49 of 65 patients) of those treated with the VLP regimen and in 84% (54 of 64 patients) of those treated with the CALASP regimen. The overall response rates were not significantly different statistically between the two treatments ($P = 0.29$) at the 5% level.

As expected, the response rate (84%) among those with a diagnosis of ALL or acute undifferentiated leukemia was significantly better regardless of treatment than the response rate (56%) among children with other cell types of acute leukemia or in the leukemic phase of lymphoma ($P = 0.04$, one-tailed test).

Documented resistance to prior therapy with VP resulted in significantly decreased response rates regardless of the treatment regimen. Thus, of those

TABLE 1.—Comparability of patients treated with VLP and CALASP regimens with respect to age, wbc count, and diagnosis

	No. of patients in treatment group—	
	VLP (%)	CALASP (%)
No. of evaluable patients	65	64
Age (yrs)		
1–2	4 (6)	3 (5)
3–5	20 (31)	16 (25)
6–10	21 (32)	29 (45)
>10	20 (31)	16 (25)
Wbc counts (cells × 10^3/mm^3)		
<10	48 (74)	47 (73)
10–99	16 (25)	17 (27)
≥100	1 (1)	0
Diagnosis*		
ALL, AUL	53 (82)	58 (91)
Lymphoma	5 (8)	4 (6)
AGL	7 (11)	2† (3)

*AUL = acute leukemia, acute undifferentiated leukemia, and acute stem cell leukemia; lymphoma = leukemic phase of lymphoma; AGL = acute granulocytic leukemia.
†One of the 2 had acute myelomonoblastic leukemia.

patients known to be resistant to VP, 65% (15 of 23) responded to VLP and 68% (13 of 19) responded to CALASP. In contrast, among those who had no documented history of resistance to VP, the response rates were 81% (34 of 42 patients) with VLP and 93% (38 of 41 patients) with CALASP. The overall response rate of 67% among those resistant to VP was significantly less ($P = 0.008$) than the 87% rate for those not resistant to VP.

In this study, 34 patients had had prior treatment with L-asparaginase. They were included in the study because they had not shown prior resistance to this agent. The response rates were very similar between those who had had no prior exposure to L-asparaginase (76 of 94 patients, 81%) and those who had had prior exposure to L-asparaginase (26 of 34 patients, 76%).

The major manifestations of toxicity were hypersensitivity with anaphylactoid reactions, hyperglycemia, pancreatitis, skin rash, and depression of bone marrow function. The incidences of these side effects are summarized in table 3. The severity of the side effects has been indicated by whether or not the therapy regimen was continued or changed (schedule change usually consisted of discontinuance of L-asparaginase). With the exception of bone marrow depression following the pulses of CA, no significant differences in toxicity were apparent between the two treatment groups.

COMMENTS

The data reported here show that these attempts to synchronize with CA the leukemic cells in S phase did not confer a therapeutic advantage when children with acute leukemia were treated with VLP. The result of treatment was measured only as the frequency with which a remission status (M_1/M_2) was achieved in the bone marrow. The degree or the "depth" of these remissions might have been reflected in the duration of these remissions. However, the maintenance treatments these children had received previously varied widely. Thus, they could not be given a uniform maintenance regimen without jeopardizing their chances of achieving a long remission. The relatively short duration of unmaintained remissions noted in patients treated with other L-asparaginase programs (1,13,14) put up ethical barriers to the consideration of maintenance without any drug treatment to measure the dura-

TABLE 2.—Response rates to VLP and CALASP by diagnosis, resistance to VP, and prior drug exposure

	Responders/No. of patients treated (%)		
	VLP	CALASP	Total
All evaluable patients	49/65 (75)	54/64 (84)	103/129 (80)
Diagnosis*			
ALL, AUL	43/53 (81)	50/58 (86)	93/111 (84)
Lymphoma	2/5 (40)	3/4 (75)	5/9 (56)
AGL	4/7 (57)	1/2† (50)	5/9 (56)
Resistance to VP			
No	34/42 (81)	38/41 (93)	72/83 (87)
Yes	15/23 (65)	13/19 (68)	28/42 (67)
Prior exposure to L-asparaginase			
No	39/49 (80)	37/45 (82)	76/94 (81)
Yes	10/16 (63)	16/18 (89)	26/34 (76)

**See* table 1 for definitions.
†The nonresponder had acute myelomonoblastic leukemia.

TABLE 3.—Incidence of significant toxic reactions

	No. of patients with reactions	
	VLP	CALASP
No. of patients	65	64
Hypersensitivity	0	4
Schedule changed	0	3
Hyperglycemia	6	3
Schedule changed	1	1
Pancreatitis	3	3
Schedule changed	2	1
Skin rash	1	0
Schedule changed	0	0
Bone marrow depression	14	35
Schedule changed	1	9

tion of disease control. Clinical data indicate that although combination therapy with L-asparaginase produces a higher response rate than L-asparaginase alone (1,13,14), the remission duration was not prolonged without maintenance therapy.

Although studies of this type may be conceptually attractive, it would appear that the practical application of kinetic approaches to clinical situations still requires considerable study (15,16). It can be speculated that in this particular investigation, some degree of synchronization may have occurred but that optimum synchronization may not have taken place at 48 hours.

It is of interest to note that other investigators utilizing bone marrow labeling index data also selected 48 hours as the optimum time for administration of cycle-specific antileukemic agents after CA synchronization (17). These authors indicate, however, that significant increases in the labeling index may occur anywhere from 24 to 96 hours after the infusion of CA (17).

In the present study, vincristine, L-asparaginase, and prednisone were started simultaneously following the CA pretreatment. Prednisone and L-asparaginase were continued daily while vincristine was given intermittently (weekly). The schedule for VP was the one used routinely and effectively in the clinical therapy for acute leukemia. Studies have indicated that the passage of cells in the cell cycle from G_1 into S phase may be blocked by both corticosteroids (18) and by L-asparaginase (19). Vincristine is maximally effective on cells during the S phase when microtubule synthesis and assembly are taking place. Therefore, it is possible that in this particular regimen some decreased effectiveness of CA synchronization resulted by limiting the numbers of cells in S phase at which time they would be maximally sensitive to vincristine.

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Randomized Prospective Trial of DTIC (NSC-45388) Alone Versus BCNU (NSC-409962) Plus Vincristine (NSC-67574) in the Treatment of Metastatic Malignant Melanoma[1,2,3]

Robert E. Bellet,* Michael J. Mastrangelo, J. Frederick Laucius, and Audley J. Bodurtha[4]

SUMMARY

Fifty patients with metastatic malignant melanoma were randomized to treatment with either DTIC (2 mg/kg/day × 10 iv) or the combination of BCNU (150 mg/m² iv) plus vincristine (VCR) (2 mg/m² iv on Day 1 only). Treatment failures were crossed over to the alternate therapy. Primary, secondary, and cumulative response rates to DTIC were 29%, 9%, and 22%, respectively. Primary, secondary, and cumulative response rates to BCNU plus VCR were 23%, 29%, and 25%, respectively. Five of 26 patients (19%) experienced objective regression from secondary therapy after failure to respond to primary therapy. DTIC produced gastrointestinal and hematologic toxic effects; BCNU plus VCR produced gastrointestinal, hematologic, and neurologic toxic effects. VCR administered at a dose of 2 mg/m² resulted in excessive neurologic toxic effects in 12 of 21 patients; a maximum VCR dose of 2 mg/injection was well tolerated by 15 subsequent patients without an adverse effect upon response rate. An analysis of tumor burden and organ involvement in responders and nonresponders suggests that DTIC is the first-choice treatment for patients with limited tumor burdens and nonvisceral metastases; BCNU plus VCR is the first-choice treatment for patients with extensive tumor burdens and visceral-predominant disease. However, failure to respond to primary therapy does not preclude response to secondary therapy with the alternate regimen.

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Since its introduction as a cancer chemotherapeutic agent, DTIC has been extensively tested in patients with metastatic melanoma. In a review of published reports, Luce (1) found 169 responders in a total of 733 patients treated, a response rate of 23%. BCNU is also effective in this disease with 22 of 123 patients (18%) responding (1). In 1970 Moon (2) reported nine responders among 20 evaluable patients treated with a combination of BCNU plus vincristine (VCR). There appeared therefore to be a significant therapeutic advantage for this combination over either DTIC or BCNU used as single agents. The purpose of this phase III study was to compare, in a randomized prospective crossover trial, the therapeutic efficacy and toxicity of DTIC alone versus the combination of BCNU plus VCR.

MATERIALS AND METHODS

Patients were acceptable for study who had histologically documented, surgically incurable, metastatic malignant melanoma and no prior treatment with chemotherapy or radiation therapy. Measurable cutaneous, subcutaneous, nodal, pulmonary, or

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[2]Supported by Public Health Service grants CA-13456 and CA-06551 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare, and by an appropriation from the Commonwealth of Pennsylvania.

[3]DTIC: CAS reg. No. 4342-03-4; imidazole-4-carboxamide, 5-(3,3-dimethyl-1-triazeno)-.

BCNU: CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-.

Vincristine: CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1).

DTIC and BCNU were supplied by the Cancer Therapy Evaluation Program, Division of Cancer Treatment, NCI.

[4]The American Oncologic Hospital and The Institute for Cancer Research Fox Chase Cancer Center, Philadelphia, Pa.

*_Reprint requests to_: Robert E. Bellet, MD, Department of Medicine, The American Oncologic Hospital, Central and Shelmire Aves, Philadelphia, Pa 19111.

hepatic metastatic disease was required for entry. Patients with a life expectancy of < 3 months were ineligible for treatment. Also ineligible for study were patients with a hemoglobin level < 10 g/100 ml, a white blood cell (wbc) count < 4000 cells/mm^3, a platelet count < 100,000 platelets/mm^3, and a blood urea nitrogen (BUN) level > 20 mg/100 ml.

Fifty consecutive eligible patients were randomized to one of two treatment regimens: DTIC alone or BCNU plus VCR. Twenty-five patients received primary therapy with DTIC (2 mg/kg/day iv for 10 consecutive days). Twenty-five patients received primary treatment with BCNU plus VCR. BCNU was administered at a dose of 150 mg/m^2 as a 1-hour iv infusion on Day 1 only. VCR was administered as an iv bolus at a dose of 2 mg/m^2 immediately after the BCNU infusion. Hemoglobin, wbc, and platelet counts were performed at 2-week intervals. BUN, serum glutamic oxaloacetic transaminase, alkaline phosphatase, and bilirubin determinations were performed on Day 28 of each cycle. Signal lesions were measured on Day 28. Patients were re-treated at 28-day intervals until tumor progression was noted, at which time they were crossed over to the alternate regimen.

Patients were considered evaluable for toxicity and response if they were followed for a minimum of 28 days after the initiation of systemic chemotherapy. There were three response categories: complete remission (CR), partial remission (PR), and no response (NR). CR indicates regression of all measurable disease resulting in no evidence of melanoma. PR is defined as a > 50% decrease in the sum of the products of the perpendicular diameters of all measurable disease for at least 1 month. NR includes all other patients evaluable for response.

Statistical analyses generating P values were derived using the Fisher's Exact Test unless otherwise specified; P values > 0.10 are reported as nonsignificant (NS). Tumor burden (g) was calculated by assuming tumor density to be 1 g/ml and tumor volume was derived from the formula $V = {}^4/_3 \times \pi \times R^3$ (R = radius).

RESULTS

Response

Objective response data are presented in table 1. There were no statistically significant differences in response rates between the treatment groups. Overall response rates for DTIC and BCNU plus VCR were 22% and 25%, respectively. Five of 26 patients (19%) experienced objective responses to secondary therapy after failure to respond to primary therapy.

Remission induction for DTIC-treated patients required 2.0 months (median) and 1.5 months (median) for CR and PR, respectively. For BCNU plus VCR responders, 1.0 month (median) was required to induce both CR and PR. Duration of remission is presented in table 2. The median durations of remission for DTIC and BCNU plus VCR responders were 12.0 and 8.0+ months, respectively. Although response duration is longer for the DTIC-treated patients, this difference is presently not significant.

In an effort to ascertain which subset of patients was most likely to respond to either DTIC or BCNU plus VCR, several clinical parameters were evaluated. There was no statistically significant difference with regard to either mean age or sex for responding or nonresponding groups to either DTIC or BCNU plus VCR (table 3). An analysis of the

TABLE 1.—Objective response

Treatment	No. of patients treated/No. evaluable*	No. of patients with—		Response rate† (%)
		CR	PR	
DTIC				
Primary	25/24	4	3	7/24 (29)
Secondary	12/12	0	1	1/12 (9)
Totals	37/36	4	4	8/36 (22)
BCNU + VCR				
Primary	25/22	4	1	5/22 (23)
Secondary	15/14	1	3	4/14 (29)
Totals	40/36	5	4	9/36 (25)

*5 patients died of metastatic melanoma within 28 days after initiation of therapy and were therefore considered unevaluable.
†Total No. of responses/No. of patients evaluable.

TABLE 2.—Response duration

Treatment	Response duration (mos)			
	CR	PR	Mean	Median
DTIC	33+, 33+, 18+, 12	12, 7+, 3, 2	15.0+	12.0
BCNU + VCR	24+, 15, 12+, 11, 6	8+, 3, 3, 2	9.3+	8.0+

TABLE 3.—Clinical profile

Treatment	Responders	Nonresponders
DTIC		
No. of patients	8	28
Sex (M/F)	4/4	14/14
Age (mean)	51	52
BCNU + VCR		
No. of patients	9	27
Sex (M/F)	4/5	12/15
Age (mean)	50	55

TABLE 4.—Relationship of tumor burden to response*

Treatment	No. of patients with tumor burden (g) of—		
	0–50	51–250	>250
DTIC	5/9 (56%)	3/9 (33%)	0/18 (0)
BCNU + VCR	5/9 (56%)	2/12 (17%)	2/15 (13%)

*No. of responses/No. of patients evaluable.

TABLE 5.—Tumor distribution and response*

Tumor site	DTIC†	BCNU + VCR‡
Non-visceral		
Skin	2/3	0/2
Subcutaneous	7/24	4/17
Lymph node	6/21	4/23
	15/48	8/42
Visceral		
Lung	1/9	3/12
Liver	0/7	0/4
Bone	0/7	0/4
	1/23	3/20

*No. of responses/No. of NR.
†87 organs involved in 36 evaluable patients.
‡73 organs involved in 36 evaluable patients.

relationship of tumor burden to response is presented in table 4. For the DTIC group, the difference in response rates between low and high tumor-burden groups was significant at the 0.003 level. For the combination of BCNU plus VCR, the difference in response rates between high and low tumor-burden groups was significant only at the 0.08 level.

Analysis of organ involvement (table 5) indicates that for DTIC ($P = 0.05$), patients with nonvisceral involvement are more likely to respond to treatment than are patients with visceral involvement. For BCNU plus VCR, there is no statistically significant difference in this regard. However, there was no improvement in hepatic or bony metastases after systemic chemotherapy for any patient with either regimen.

The median survival times from initiation of chemotherapy are presented in table 6. Statistical analysis was calculated by the Mann-Whitney U Test. There was a statistically significant prolongation of survival for all responders versus all nonresponders for both DTIC ($P = 0.007$) and BCNU plus VCR ($P = 0.002$). The survival for female responders was not significantly longer than the survival for male responders in either treatment regimen. Although the median survival for nonresponding women exceeded the median survival for nonresponding men in both treatment categories, the difference was not significant. Finally, no difference in survival was noted between the DTIC and BCNU plus VCR responders.

Toxicity

Drug-induced toxicity for each treatment regimen is presented in table 7. DTIC produced gastrointestinal and hematologic toxic effects; BCNU plus VCR produced gastrointestinal, hematologic, and neurologic toxic effects. No hepatic, renal, pulmonary, or cardiac toxic effects were noted in patients receiving either treatment.

Repeated courses of therapy with DTIC could be administered at monthly intervals without cumulative hematologic toxicity. However, for the combination of BCNU plus VCR, delays in treatment or dose reduction were necessitated in three of 14 patients

TABLE 6.—Survival*

	DTIC		BCNU + VCR	
	No. of patients	Median survival (mos)	No. of patients	Median survival (mos)
Men				
Responders	4	20.5+	4	14.5+
Nonresponders	14	4.5	12	4.5
Total	18	5.5	16	5.5
Women				
Responders	4	19.5+	5	17.0
Nonresponders	14	10.5	15	7.0
Total	18	11.0	20	10.0+
Total				
Responders	8	19.5+	9	15.0+
Nonresponders	28	6.5	27	4.5
Patients	36	8.0+	36	6.0

*From initiation of chemotherapy.

TABLE 7.—Toxicity (first course of therapy)

Toxic effects	DTIC*	BCNU + VCR*
Hematologic		
Leukopenia (<2000 cells/mm³)	0/36	0/36
Thrombocytopenia (<100,000 cells/mm³)	1/36	4/36
Anemia	0/36	0/36
	1/36	4/36
Gastrointestinal		
Nausea	23/26	25/36
Emesis	12/36	16/36
Diarrhea	0/36	0/36
	23/36	25/36
Neurologic		
VCR (2 mg/m²)	—	12/21
VCR (2 mg)	—	3/15
	0/36	15/36

*No. of patients with toxic reactions/No. of evaluable patients.

receiving three or more courses of therapy because of persistent thrombocytopenia.

When given the full dose of VCR (2 mg/m²), significant neurologic toxic effects occurred in 12 of 21 patients. The most consistent manifestations were jaw and throat pain, which were generally well controlled with analgesics. However, a frequent complication was severe constipation which was most difficult to treat. For this reason, the next 15 patients received a maximum VCR dose of 2 mg/injection. Only three of 15 patients treated with this dose had neurologic toxic reactions. Of the nine

patients responding to the combination of BCNU plus VCR, five of 15 patients (33%) responded who received the 2-mg maximum dose and four of 21 patients (19%) responded who received the full dose of 2 mg/m². Limiting the dose of VCR to 2 mg/injection did not therefore adversely affect response rate.

DISCUSSION

DTIC used as a single agent and the combination of BCNU plus VCR are among the most effective chemotherapeutic regimens in the treatment of metastatic melanoma. In an attempt to gain greater insight into the spectra of activity of these two regimens, a prospective randomized crossover study was initiated.

DTIC produced a 24% response rate in melanoma when administered at a dosage of 4.5 mg/kg/day × 10 consecutive days iv (1). In this study, a dose of 2.0 mg/kg/day for 10 consecutive days iv resulted in a 22% response rate with mild gastrointestinal toxicity and minimal hematologic toxicity. These data are consistent with those of Nathanson et al (3) who demonstrated that low-dose DTIC (2 mg/kg/day) was as effective as the 4.5-mg/kg/day dose, with an attendant significant decrease in toxicity.

Moon et al (2,4) have treated a series of melanoma patients with BCNU (150 mg/m²) plus VCR (2 mg/m²). In their studies, neurologic toxicity was not sufficient to warrant reduction in the VCR dose. This is in contrast to our experience in which significant neurologic toxicity prompted us to limit the VCR dose to 2 mg/treatment.

Moon et al (2,4) have reported response rates of 45% and 16%, respectively, for the combination of BCNU plus VCR in patients with metastatic melanoma. In a compilation of reported and unpublished data Luce (1) found 28 responders of 122 patients treated, for a response rate of 23%. The overall response rate for this combination in the present series is 25%. The variation in response rates is doubtless the result of several factors including clinical characteristics of patient population, chance, and dose and schedule modifications.

This phase III study reveals no statistically significant difference in overall response rates between DTIC (22%) and BCNU plus VCR (25%). In addition, there was no statistically significant difference in response rates when comparing primary therapy with DTIC versus primary therapy with BCNU plus VCR. The latter observation is consistent with the findings of Moon et al (4) in a randomized prospective trial comparing BCNU plus VCR with two DTIC regimens; no significant differences in primary response rates between any treatment arms were noted.

In our study there was no significant difference in response rates between secondary therapy with DTIC and secondary therapy with BCNU plus VCR. Furthermore, there was no statistically significant difference between the primary and secondary response rates for either regimen. Therefore, DTIC and BCNU plus VCR are equally effective as either primary or secondary therapy for metastatic malignant melanoma. This observation is in contrast to the statement of Moon et al (4) that "there were no definite responses in any of the patients crossed over to the alternate drug regimens." In addition, Ahmann et al (5) state that "secondary treatment in disseminated malignant melanoma is of little or no benefit." Our finding that five of 26 patients (19%) who failed one primary treatment regimen but responded to secondary therapy is supported by the preclinical studies of Kline et al (6) who demonstrated that a line of murine L1210 leukemia resistant to DTIC was sensitive to BCNU. A melanoma patient who fails to respond to primary therapy should not therefore be considered chemotherapy-resistant. In fact, one patient in our present series has achieved a CR of > 1 year's duration with BCNU plus VCR after having failed DTIC therapy.

Although we note no statistically significant differences between DTIC and BCNU plus VCR in primary, secondary, or cumulative response rates, important information can be derived from tables 4 and 5. For DTIC, there is a significant ($P = 0.003$) difference in the response rates between low and high tumor-burden groups. For BCNU plus VCR, the difference in response rates is significant only at the 0.08 level. An analysis of organ involvement reveals a significant difference in response to DTIC ($P = 0.05$) in favor of nonvisceral metastases. For BCNU plus VCR, there is no statistically significant difference in this regard. We therefore consider DTIC (on the basis of more modest toxicity) the first-choice treatment in patients with limited tumor burdens and nonvisceral metastases; BCNU plus VCR is the first-choice treatment in patients with extensive tumor burdens and visceral-predominant metastases.

Finally, Luce (1) suggests that "femaleness combined with chemotherapy" improves survival in patients with metastatic melanoma. Our data do not substantiate this contention. We found no significant difference in survival between men and women who responded to either DTIC or BCNU plus VCR. Furthermore, there was no significant difference in survival when comparing men and women who did not respond to either treatment regimen.

In summary, we conclude that:

a. DTIC and BCNU plus VCR are equally effective as either primary or secondary therapy for metastatic melanoma;

b. failure to respond to primary therapy does not preclude response to secondary therapy with the alternate regimen;

c. DTIC (on the basis of more modest toxicity) is the first-choice treatment in patients with limited tumor burdens and nonvisceral metastases;

d. BCNU plus VCR is the first-choice treatment in patients with extensive tumor burdens and visceral metastases;

e. VCR at a dose of 2 mg/m^2 produces significant neurologic toxic effects; limiting the dose of VCR to 2 mg/injection reduces toxicity and does not adversely affect response rate;

f. there is a significant prolongation of survival for all responders versus all nonresponders to both DTIC and BCNU plus VCR;

g. "femaleness plus chemotherapy" does not improve survival.

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DTIC (NSC-45388) and Combination Therapy for Melanoma. I. Studies With DTIC, BCNU (NSC-409962), CCNU (NSC-79037), Vincristine (NSC-67574), and Hydroxyurea (NSC-32065)[1,2,3]

R. Davilene Carter,* Edward T. Krementz, George J. Hill II, Gerald E. Metter, William S. Fletcher, Frederick M. Golomb, Theodor B. Grage, John P. Minton, and Frank C. Sparks[4,5]

SUMMARY

A total of 270 patients with metastatic malignant melanoma were entered into a randomized chemotherapy study conducted by the Central Oncology Group (COG) over a period of 2 years (COG protocol No. 7130). The study utilized DTIC, CCNU, BCNU, vincristine, and hydroxyurea. The results of therapy with DTIC alone were compared with three combinations: (*a*) DTIC, CCNU, and vincristine; (*b*) DTIC, BCNU, and vincristine; and (*c*) DTIC, BCNU, and hydroxyurea. There were 243 evaluable patients out of 270. The response rate was 17.3% (42 of 243 patients) for evaluable patients and 15.5% (42 of 270 patients) for all patients entered in the study. The results showed no statistically significant difference in the response rates among the four treatment arms. However, several significant points were observed: (*a*) A 13% response rate was obtained using a combination of agents that included DTIC in patients who had previously shown no response to DTIC used as a single agent. (*b*) There was a significant difference in survival time when comparing responders to those with no change and to those with progression. (*c*) Toxicity was noted to be greater in responders than in nonresponders. (*d*) Two of the four treatment arms were considered most advantageous due to the ease of administration. These treatment arms were DTIC, BCNU, and vincristine and DTIC, CCNU, and vincristine administered in 5-day courses every 6 weeks. (*e*) The percentage of response and length of survival were significantly greater in patients without brain or liver metastasis. (*f*) In comparing men to women there was no statistically significant difference in response rates or durations of response. (*g*) There was no statistically significant difference in survival when comparing site of primary lesion.

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[2]Supported by Public Health Service grants CA-12284 and CA-12271 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3]DTIC: CAS reg. No. 4342-03-4; imidazole-4-carboxamide, 5-(3,3-dimethyl-1-triazeno)-.

BCNU: CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-.

CCNU: CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-.

Vincristine: CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1).

Hydroxyurea: CAS reg. No. 127-07-1.

DTIC, BCNU, CCNU, and hydroxyurea were supplied by Cancer Therapy Evaluation Program, Division of Cancer Treatment, NCI.

[4]This study was performed by the Central Oncology Group (COG protocol No. 7130). Authors are from the following institutions: Tulane University, New Orleans, La (*R. D. Carter and E. T. Krementz*); Washington University, St. Louis, Mo (*G. J. Hill II*); University of Wisconsin, Madison (*G. E. Metter*); University of Oregon, Portland (*W. S. Fletcher*); New York University Medical Center, New York (*F. M. Golomb*); University of Minnesota, Minneapolis (*T. B. Grage*); Ohio State University, Columbus (*J. P. Minton*); and University of California, Los Angeles (*F. C. Sparks*). Other participating institutions were: American Oncologic Hospital, Philadelphia, Pa (*J. Strawitz*); Bronx Lebanon Hospital Center, NY (*M. R. Azelrod*); University of California, Davis (*N. C. Andrews*); University of Colorado, Denver (*R. E. Gerner*); Cooper Hospital, Cherry Hill, NJ (*N. Siegel*); Duke University Medical Center, Durham, NC (*W. W. Shingleton*); COP Hawaii, Honolulu (*J. P. Keenan*); University of Illinois, Chicago (*T. K. Das Gupta*); Jefferson Medical College, Philadelphia, Pa (*A. J. Weiss*); University of Kentucky, Lexington (*W. R. Meeker*);

From 1972 to 1974 the Central Oncology Group (COG) undertook a prospective randomized study using four different treatment programs for patients with metastatic malignant melanoma who had measurable lesions. The four treatment programs were: (*a*) DTIC alone (D), (*b*) DTIC plus CCNU and vincristine (DCV), (*c*) DTIC plus BCNU and vincristine (DBV), and (*d*) DTIC plus BCNU and hydroxyurea (DBH). Each combination of agents used in this study had previously been investigated in individual phase I, phase II, or pilot studies (1,2).

DTIC has been the most useful single agent in the treatment of malignant melanoma, with a 20%–25% response rate and tolerable toxicity in most studies (3–6). In COG phase I and II studies only one drug-related death occurred in 506 patients who received doses as high as 4.5 mg/kg/day for 10 days. The nitrosoureas have previously been reported to produce responses in melanoma (7–10). A 10%–20% response rate with hydroxyurea has been reported but the best treatment schedule has not yet been determined (11).[6] Vincristine has also been reported to be useful in the treatment of melanoma (12–14).

This study was designed to see if an improved response rate could be attained with DTIC in combination therapy compared with DTIC alone. The different drugs and combinations were selected because of differences in mechanism of action, ease of administration, and variations in time and type of toxicity.

MATERIALS AND METHODS

Patients

Patients with metastatic malignant melanoma with measurable systemic and/or regional disease who could not be controlled by other measures were eligible for entry in the study. Patients previously treated with the agents used in this study (except for DTIC), either systemically or regionally, were ineligible. The patients also must have (*a*) been between 15 and 80 years of age, (*b*) had adequate bone marrow reserve, and (*c*) had good liver function (bilirubin level < 2 mg/100 ml). Patients who had received radiation therapy > 3000 rads to the pelvis or lumbar spine were ineligible. No prior therapy within the previous 4 weeks was allowed.

Fourteen of the 27 cooperating institutions involved in this study contributed ten or more patients.

Dosage Schedule

The dosage schedules are shown in figure 1. The doses were selected on the basis of previous phase I or pilot studies. Patients who had previously received DTIC, or those who were randomized to receive DTIC alone initially, were eligible to receive one of the combination-therapy programs following progression on DTIC.

Evaluation Procedure

After a 6-week minimum adequate trial the patients were evaluated according to the following response criteria:

a. CR—The disappearance of all objective and subjective evidence of tumor.

b. PR—A decrease of 50% or more in the products of the diameter of lesions measured or 20% in the number of lesions being counted, or disappearance of objective evidence of tumor in sites that are being measured when tumor remains in nonmeasured sites. In patients with liver metastasis the criteria were a 50% decrease in the liver size and/or a 50% improvement in the liver-function profile.

c. No change (*NC*)—An increase or decrease of $< 50\%$ of the products of the diameter of measurable lesions or a $\leq 10\%$ change in numbers of lesions being counted.

d. Progression—A 50% or more increase in the products of the diameter of the lesions, the appearance of new lesions, or a $\geq 10\%$ increase in the lesions being counted. In patients with liver involvement progression was defined as a 50% increase in the liver size and/or a 50% worsening or shift to abnormal in the liver-function profile.

In addition to response all patients were evaluated for length of survival and for toxicity. Toxicity parameters included nausea and vomiting, diarrhea, leukopenia, thrombocytopenia, and neurotoxicity. Grades 3 and 4 were the most severe (severe nausea

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Latter-Day Saints Hospital, Salt Lake City, Utah (*S. J. Altman*); Medical Arts Clinic, Farmington, Mo (*R. C. Hoye*); New Jersey College of Medicine, Newark (*B. F. Rush, Jr*); Roswell Park Memorial Institute, Buffalo, NY (*E. G. Elias*); St. John's Hospital, Santa Monica, Calif (*W. L. Wilson*); St. Vincent's Hospital, New York, NY (*T. F. Nealon*); University of Texas, San Antonio (*J. B. Aust*); Toledo Clinic, Inc, Ohio (*C. D. Cobau*); Wilmington Medical Center, Del (*R. W. Frelick*); and University of Wisconsin, Madison (*G. Ramirez*).

[5]We wish to acknowledge the expert technical assistance of Ms. Kathleen Rebstock and Mr. Scot Moss.

[6]Shaw R, and Bruner J. Clinical brochure for hydroxyurea. New Brunswick, NJ, The Squibb Institute for Medical Research, 1968.

**Reprint requests to*: R. Davilene Carter, MD, Department of Surgery, Tulane University School of Medicine, 1430 Tulane Ave, New Orleans, La 70112.

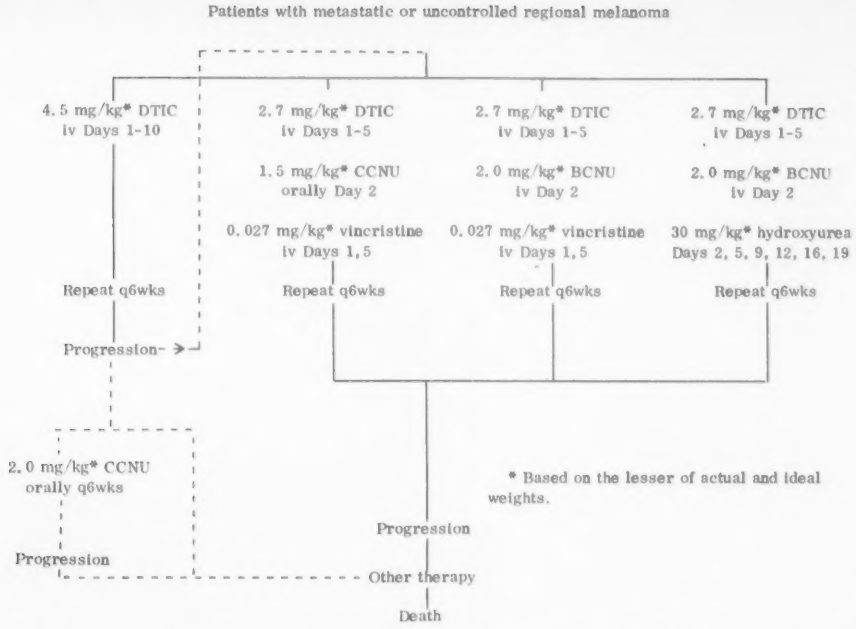
Patients with metastatic or uncontrolled regional melanoma
4.5 mg/kg* DTIC iv Days 1-10
2.7 mg/kg* DTIC iv Days 1-5
1.5 mg/kg* CCNU orally Day 2
0.027 mg/kg* vincristine iv Days 1,5
2.7 mg/kg* DTIC iv Days 1-5
2.0 mg/kg* BCNU iv Day 2
0.027 mg/kg* vincristine iv Days 1,5
2.7 mg/kg* DTIC iv Days 1-5
2.0 mg/kg* BCNU iv Day 2
30 mg/kg* hydroxyurea Days 2, 5, 9, 12, 16, 19
Repeat q6wks
Repeat q6wks
Repeat q6wks
Repeat q6wks
Progression
2.0 mg/kg* CCNU orally q6wks
Progression
* Based on the lesser of actual and ideal weights.
Progression
Other therapy
Death

FIGURE 1.—COG protocol No. 7130—DTIC melanoma combination.

and vomiting, white blood cell [wbc] count < 2000 cells/mm^3, or platelet count < 75,000 cells/mm^3). (Anemia resulted from either the drug or disease.)

Statistical Methods

Patients were randomized in a manner which would evenly distribute the four treatment arms by previous treatment, stage (stage II—regional disease; stage III—systemic disease), and organ involvement.

The statistical methods used included: (*a*) chi^2 test to test differences in response rates; (*b*) Gehan's generalization of the Wilcoxon test (15) or Breslow's generalization of the Kruskal-Wallis test (16) for comparing duration of response and survival curves; (*c*) the Kruskal-Wallis test corrected for ties[7] comparing response evaluations by the level of toxicity; and (*d*) Kaplan and Meier methods (17) for calculating response and survival curves.

RESULTS

A total of 270 patients were entered in this study and 243 (90%) were considered evaluable. Patients were grouped into two prior treatment categories: those with no prior DTIC therapy (212 patients) and those who had previously received DTIC therapy (31 patients). Of those who had previously been treated with DTIC, 14 had received the same schedule used in the DTIC-alone arm of this study. The 27 unevaluable patients were evenly distributed among the various treatment arms and were excluded for the following reasons: death prior to the end of a 6-week trial period, loss to followup, or incomplete followup. There were eight deaths within 21 days after therapy was started because of either massive intracerebral hemorrhage or progressive metastatic melanoma. No deaths occurred from drug toxicity.

RESPONSE

In table 1 the results are shown for all evaluable patients by treatment in absolute numbers and as response rates. For those patients receiving treatment D (DTIC alone), eight of 48 (16%) responded (three CRs), for those receiving DCV, 11 of 67 (16%) responded (two CRs), for those receiving DBV, 15 of 64 (23%) responded (no CRs), and for those receiving DBH, eight of 63 (13%) responded (three CRs). Responses thus occurred in 42 patients, with a response rate of 15.5% of the 270 study patients and 17.3% of the 243 evaluable patients.

The median durations of response in the group

[7]Klotz J. One-way layout for counts and the exact H test with ties. Madison, University of Wisconsin Department of Statistics, Technical Report 407, 1975.

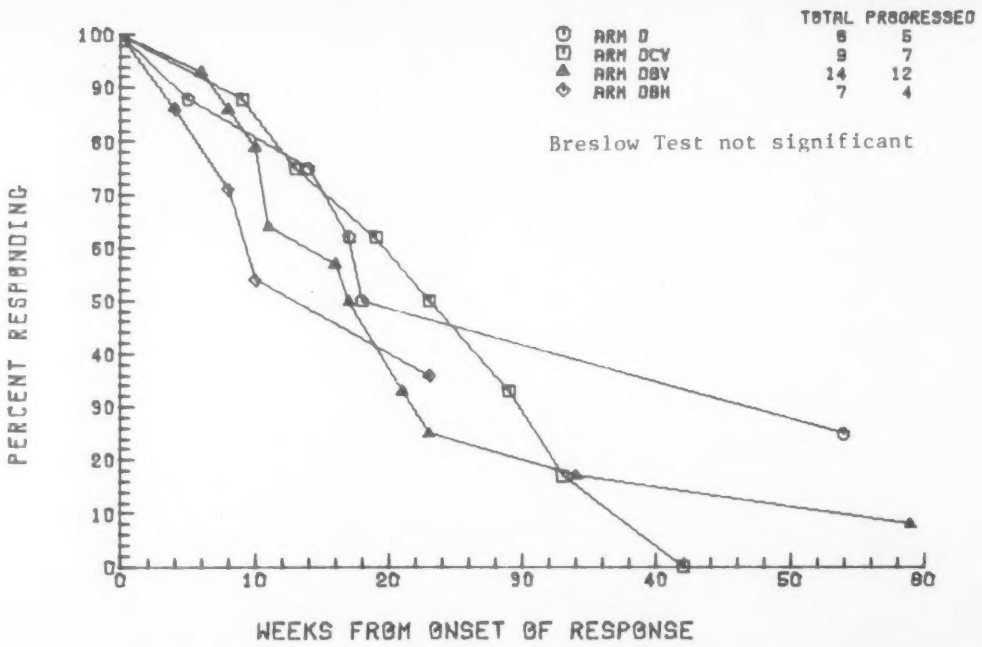

FIGURE 2.—Patients with no prior DTIC: duration of response by treatment.

with no prior therapy were: 18 weeks (treatment D), 23 weeks (treatment DCV), 17 weeks (treatment DBV), and 13 weeks (treatment DBH). These differences are not significant. The duration of response curves are shown in figure 2.

The 31 patients previously treated with DTIC were randomized to one of the three combination-therapy arms following progression. There were four PRs on combination therapy; the results are presented by treatment arm in table 2. Of the four patients who responded, two had shown NC and two had progressed on DTIC therapy alone.

There was no statistically significant difference in response rates among patients of various age groups. In considering the response according to sex the rates ranged from 18% to 27% for women and from 8% to 20% for men. These variations are not statistically significant by treatment ($P \simeq 0.16$) or by sex ($P \simeq 0.19$) (fig 3).

The patients were randomized with regard to those who had brain or liver involvement or those who had neither; only 14 patients were entered with brain involvement, a number too small for statistical evaluation. Thirty-nine patients with liver involvement were randomized into the four treatment arms. Of these there were no responses on treatment arm D; on the remaining arms, two of ten responded to DCV, two of 13 to DBV, and one of

TABLE 1.—Summary of responses for evaluable patients

	Patient responses									
	CR		PR		NC		Progression		Total	
Treatment	No.	%	No.	%	No.	%	No.	%	No.	%
D	3	6	5	10	21	44	19	40	48	100
DCV	2	3	9	13	27	40	29	43	67	99
DBV	0	0	15	23	23	35	27	42	65	100
DBH	3	5	5	8	31	49	24	38	63	100
Totals	8	3	34	14	102	42	99	41	243	100

TABLE 2.—Summary of responses for patients previously treated with DTIC

	Patient responses							
	PR		NC		Progression		Total	
Treatment	No.	%	No.	%	No.	%	No.	%
DCV	2	18	3	27	6	55	11	100
DBV	1	10	4	40	5	50	10	100
DBH	1	10	5	50	4	40	10	100
Totals	4	13	12	39	15	48	31	100

eight to DBH. An additional eight patients with liver involvement who had previously received DTIC and had progressed were randomized to a combination-treatment arm. Of these, six patients continued to progress and two showed NC. Again, the numbers are too small to be conclusive. Patients were randomized by stage; all were stage III except for 17 stage II patients. In these 17 there was one response.

Survival

No statistically significant difference in survival was observed between patients evaluated by disease stage and the results are shown for the combined group of patients. The overall survival curves show no statistically significant difference by treatment arms in patients with no prior DTIC therapy (fig 4). There is also no statistically significant difference in survival between the three treatment arms for patients who had previously received DTIC.

When survival is examined by patient response, there is a highly significant difference ($P << 0.001$) comparing response versus NC versus progression, with median survivals of 65, 33, and 16 weeks respectively (fig 5). When examined by individual treatment type, this same gradient was observed ($P < 0.001$ in each arm).

As expected, patients without brain and liver involvement survived longer than those with involvement of those organs (fig 6). Although patients were not randomized by primary site, no significant difference was observed in survival by primary site.

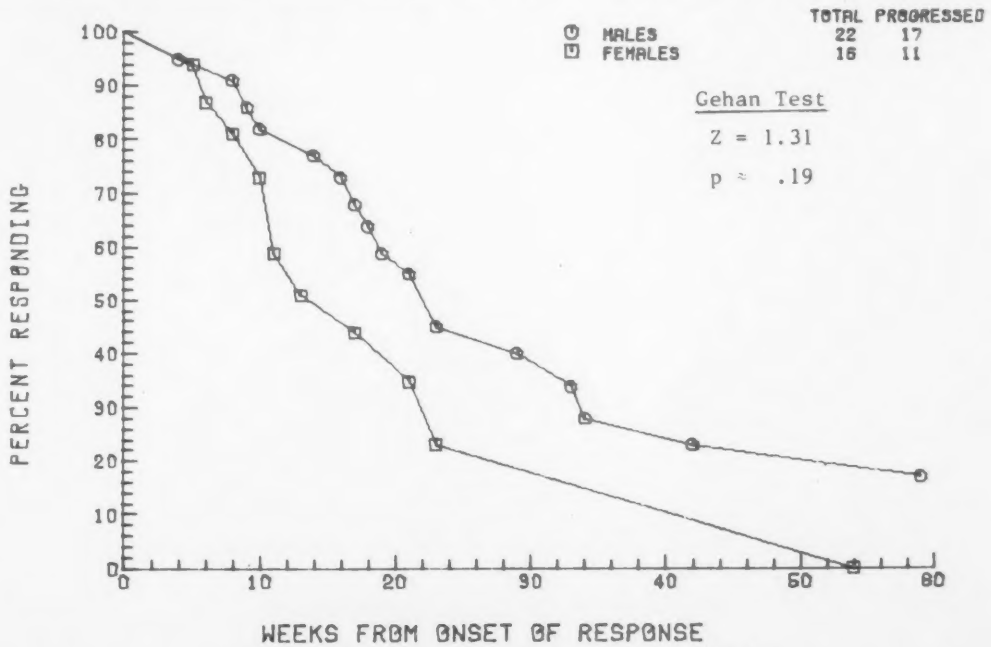

FIGURE 3.—Patients with no prior DTIC: duration of response by sex.

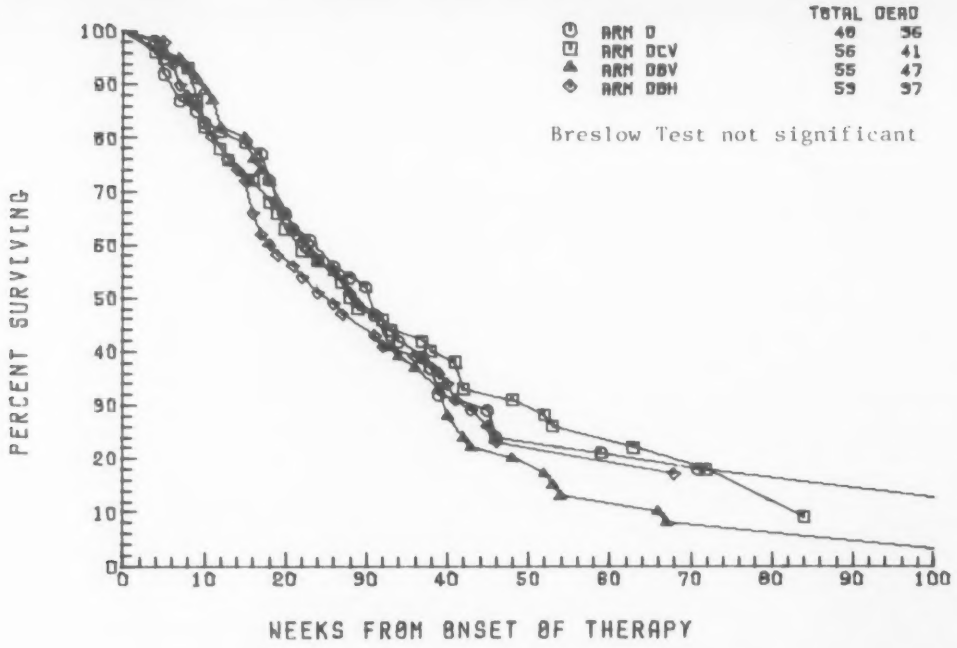

FIGURE 4.—Patients with no prior DTIC: survival by treatment.

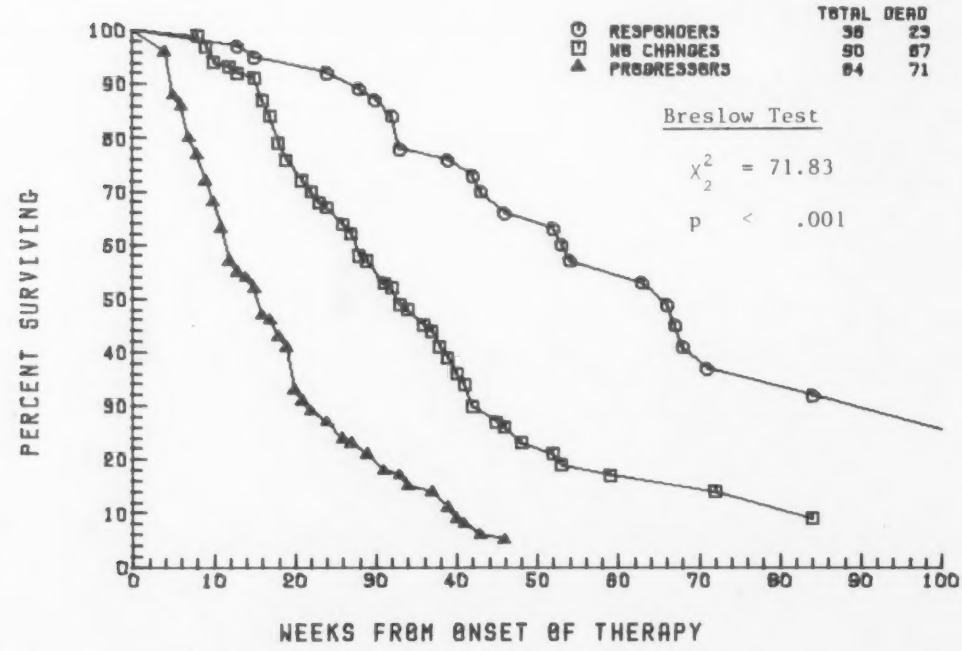

FIGURE 5.—Patients with no prior DTIC: survival by response.

Toxic Effects

A summary of toxic effects is shown by treatment in table 3. Ten percent to 16% of the patients treated experienced severe nausea and vomiting. No patient had a wbc count < 1000 cells/mm^3 and only 2% had a wbc count < 2000.

Thrombocytopenia occurred in all treatment arms but was low in incidence and severity with DBV. A total of 22 patients had mild neurotoxicity: 21 who received either of the two arms with vincristine and one who received DBH. Only two patients had alopecia.

In examining response rates by toxicity, all of the responders had some toxic effects. With regard to leukopenia the patients having some degree of toxicity (wbc count ≤ 4000) had a significantly higher response rate than those experiencing no toxic effects on all treatment arms except DBH. In patients with no prior DTIC the leukopenic patients had a 35% response rate compared to an 8% response rate in nonleukopenic patients. When considering the categories of response (CR, PR, NC, progression), the difference was significant at $P < 0.001$. In relation to survival, leukopenic patients survived longer than nonleukopenic patients as seen in figure 7 ($P < 0.002$), with a median survival of 41 versus 22 weeks.

DISCUSSION

This study demonstrated that response rates with DTIC alone or in the combinations selected were somewhat < 20%, and there was little difference in the overall response rates between DTIC alone versus DTIC in combination with a nitrosourea and vincristine or hydroxyurea. Nevertheless, it was seen that failure to respond to DTIC did not preclude a subsequent response to a combination that included DTIC.

There was an interesting group of 102 patients who had NC. This category of evaluation appears to be important because although these patients did not have as long a survival time as the responders, their survival was significantly longer than the progressors.

It appears that once a patient has developed metastatic disease, the site of the original tumor does not influence the course of the disease or the response rate.

The results of our study compared with those of two other studies using a similar dosage schedule (18,19) were similar with respect to response and toxicity. When comparing our results to a third study which utilized a higher dosage schedule (20) we did not achieve such a good response, nor was our toxicity as severe as in their study. This sug-

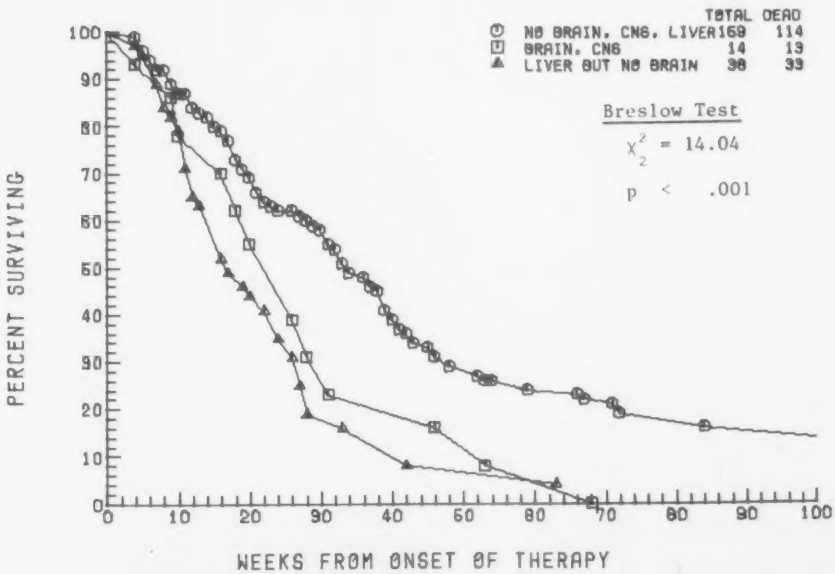

FIGURE 6.—Patients with no prior DTIC: survival by organ involvement.

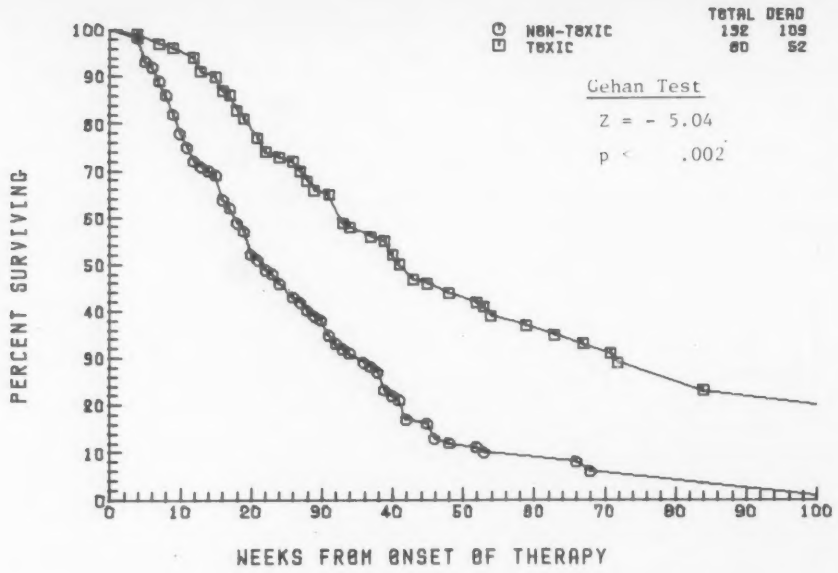

FIGURE 7.—Patients with no prior DTIC: survival by leukopenia.

TABLE 3.—COG protocol No. 7130: summary of toxic effects*

	Patients with toxicity: grade—												Treatment day of greatest severity	
	0		1		2		3		4		Total			
Toxic effect	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	Median	Range
Nausea and vomiting†														
D	6	12	15	31	19	40	6	12	2	4	48	99	2	1–106
DCV	12	18	24	36	22	33	9	13	0	0	67	100	1	1–349
DBV	18	28	16	25	24	37	4	6	3	5	65	101	2	1–142
DBH	14	22	19	30	25	40	4	6	1	2	63	100	1	1–359
Diarrhea†														
D	45	94	2	4	1	2	0	0	0	0	48	100	27	5–173
DCV	62	93	2	3	3	4	0	0	0	0	67	100	11	2–49
DBV	59	91	5	8	1	2	0	0	0	0	65	101	14	1–512
DBH	61	97	2	3	0	0	0	0	0	0	63	100	—	—
Leukopenia‡														
D	35	73	8	17	3	6	2	4	0	0	48	100	49	5–127
DCV	35	52	21	31	8	12	3	4	0	0	67	99	58	4–682
DBV	33	51	19	29	12	18	1	2	0	0	65	100	45	5–360
DBH	42	67	12	19	9	14	0	0	0	0	63	100	85	5–552
Thrombocytopenia‡														
D	34	71	6	12	2	4	2	4	4	8	48	99	40	12–303
DCV	55	82	7	10	2	3	1	1	2	3	67	99	29	5–342
DBV	50	77	9	14	6	9	0	0	0	0	65	100	47	16–296
DBH	41	65	7	11	8	13	3	5	4	6	63	100	47	8–293

*Variations in percentages due to rounding error. Anemia (due to drug or disease) was experienced by 15% of the evaluable patients receiving DTIC alone, 16% of the patients receiving DCV, 20% of the patients receiving DBV, and 16% of the patients receiving DBH. Anemia = a depressed hemoglobin level (≤ 10 g/100 ml) or hematocrit value (≤ 30%) due to either drug or disease.

†Grades generally reflect the following: 0 = no toxicity; 1 = mild; 2 = moderate; 3 = severe; 4 = life threatening.

‡Grades for hematologic toxicities (cells/mm³) are as follows: 1 = wbc count 3000–4000, platelet count 100,000–125,000; 2 = wbc count 2000–2999, platelet count 75,000–99,999; 3 = wbc count 1000–1999, platelet count 50,000–74,999; 4 = wbc count <1000, platelet count <50,000.

gests that in order to elicit the best response rate a high enough drug dose should be given to produce mild to moderate leukopenia, and with this in mind we have undertaken a new study (COG protocol No. 7131) which utilizes escalated doses in order to produce intentional toxicity. This new study has eliminated the DBH treatment arm, a complex therapeutic program which produced no better overall response rates or survival than the simpler programs. The treatment arms in COG protocol No. 7131 are: (*a*) DTIC alone (4.5 mg/kg × 10 days); (*b*) DTIC (2.7 mg/kg × 5 days) plus vincristine (0.027 mg/kg, Days 1 and 5) plus CCNU (2.0 mg/kg orally, Day 2); (*c*) DTIC (2.7 mg/kg × 5 days) plus vincristine (0.027 mg/kg, Days 1 and 5) plus BCNU (3 mg/kg iv, Day 2); and (*d*) DTIC (4.5 mg/kg × 5 days) plus vincristine (0.027 mg/kg, Days 1 and 5) plus BCNU (2 mg/kg iv, Day 2).

Patients who fail to achieve either a CR or mild leukopenia (< 4000 cells/mm^3) or thrombocytopenia (< 100,000 cells/mm^3) receive dose escalations of 20% in each subsequent course unless other severe toxicity occurs. This study is in its initial phase and has not yet been subjected to statistical analysis.

COG protocol No. 7130 was designed so that one of the treatment arms (DBV) would be similar to an arm included in a randomized combination-chemotherapy regimen undertaken by the World Health Organization Collaborating Centres for Evaluation of Methods of Diagnosis and Treatment of Melanomas. The results of their investigation have been published (21).

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Phase I Evaluation of Dianhydrogalactitol (NSC-132313)[1,2,3]

Charles D. Haas, Ronald L. Stephens, Marty Hollister, and Barth Hoogstraten[4,*]

SUMMARY

A toxicologic evaluation of dianhydrogalactitol in man was completed for a 5- and a 10-day schedule. The maximum tolerated dose was 30 mg/m²/day for the 5-day schedule and 21 mg/m²/day for the 10-day schedule. Dose-limiting myelosuppression occurred with both schedules, with leukopenia and thrombocytopenia being observed at median Days 15 and 19 respectively for the 5-day course and median Days 22 and 26 for the 10-day course. Nausea was infrequent and mild. Responses were obtained in one patient with laryngeal carcinoma and in one patient with adenocarcinoma of the lung.

[Cancer Treat Rep 60:611–614, 1976]

Dianhydrogalactitol (DAG) is the principal product resulting from the treatment of dibromodulcitol (DBD)[5] with mild alkali or normal human serum (1,2). Figure 1 shows the structure of DAG. These two drugs are thought to function as alkylating agents (3,4). DAG is more effective than DBD in inhibiting DNA, RNA, and protein synthesis and is more effective in cross-linking DNA (5,6). In vitro data derived from the treatment of synchronized HeLa cells at varying times within the cell cycle show that DAG is maximally active in the G_1 and late S-early G_2 phases (7).

DAG has shown activity in a large number of animal tumors including L1210 and P388 leukemias; B16 melanoma; NK/Ly, Ehrlich, Sarcoma 180, and Yoshida ascites tumors; Walker 256 and Guérin carcinomas; and transplanted myeloid leukemia (8).[6] It is the most active agent to date against the murine intracranial ependymoblastoma and has been considerably more effective at equitoxic doses than DBD against L1210 leukemia.

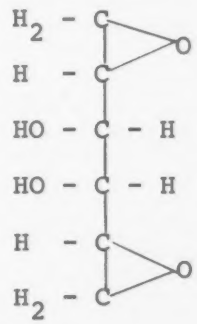

FIGURE 1.—Structure of DAG.

Dose-limiting myelosuppression was observed in the dog and monkey, the toxic dose low being 6 mg/m²/day × 5 days.[6] Gastrointestinal, hepatic, and renal toxic effects occurred only with the highest doses of drug. Pharmacokinetic and distribution studies using ^{3}H-DBD and ^{3}H-DAG labeled at position 1 indicate that renal excretion may be the major route of elimination of DAG or its metabolites (9).

MATERIALS AND METHODS

DAG was supplied in lyophilized form in 50-mg vials. The drug is dissolved in 5 ml of sterile water for injection; this yields a clear, colorless solution with each milliliter containing 10 mg. Injections

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[2]Supported by contract N01-CM-43802 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare (DHEW).

[3]Dianhydrogalactitol: CAS reg. No. 23261-20-3; galactitol, 1,2:5,6-dianhydro-. Supplied by Cancer Therapy Evaluation Program, DCT, NCI.

[4]Division of Clinical Oncology, University of Kansas Medical Center, Kansas City.

[5]Dibromodulcitol: NSC-104800; CAS reg. No. 10318-26-0; galactitol, 1,6-dibromo-1,6-dideoxy-.

*_Reprint requests to:_ Barth Hoogstraten, MD, Division of Clinical Oncology, University of Kansas Medical Center, Rainbow Blvd at 39th St, Kansas City, Kan 66103.

[6]Handelsman H. Preclinical brochure: 1,2:5,6-dianhydrogalactitol (NSC-132313). Prepared by the NCI, Bethesda, Md, May 1974.

were given through a running iv by rapid injection within 30 minutes after reconstitution.

The starting dose for both the 5- and 10-day schedules was 2 mg/m²/day. Dose escalation was performed via a modified Fibonacci search scheme. Three or more patients were studied at each dose level; if at least stable disease was noted and no toxicity had been encountered, dose escalations were used in the same patient.

Patients were considered eligible for treatment if they had:

(*a*) histologically proven malignant disease not amenable to conventional forms of therapy;
(*b*) no chemotherapy and/or radiotherapy for at least 4 weeks previously (8 weeks previously with nitrosoureas) and complete recovery from all toxic effects of prior treatment;
(*c*) adequate bone marrow reserve;
(*d*) adequate hepatic and renal function with a serum bilirubin and creatinine value <2.0 mg/100 ml;
(*e*) a disease status which would permit an observation period of at least 2 months; and
(*f*) signed, informed consent in keeping with guidelines of the DHEW and the institutional Human Experimentation Committee.

Pretreatment evaluation included a complete medical history and physical examination, bone marrow aspiration, complete blood cell (cbc) count with differential, platelet count, blood urea nitrogen, serum creatinine, serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, serum alkaline phosphatase with heat fractionation, serum bilirubin, total protein and albumin, prothrombin time, chest roentgenogram, urinalysis, and other tests as indicated. Performance status, weight, and extent of disease were also documented.

TABLE 1.—Hematologic toxicity grading criteria

Toxicity grade	Leukopenia (cells/mm³)	Thrombocytopenia (platelets/mm³)
0	>4000	>150,000
1	3000–3999	75,000–149,999
2	2000–2999	50,000–74,999
3	1000–1999	25,000–49,999
4	<1000	<25,000

During treatment, cbc and platelet counts were checked weekly while the other tests and determinations included in the pretreatment evaluation were performed every 3 weeks. A bone marrow reexamination was performed when indicated. Hematologic toxicity criteria are shown in table 1.

Responses are defined as follows: *complete response (CR)*—disappearance of all known disease

TABLE 2.—DAG toxicity for the 5- and 10-day course

DAG dose (mg/m²/day)	No. of patients with— Leukopenia (grade)					Thrombocytopenia (grade)					Nausea and vomiting* (grade)				
	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
5-day course															
2	2	1†	–	–	–	2	1†	–	–	–	3	–	–	–	–
4	3	–	–	–	–	3	–	–	–	–	3	–	–	–	–
6	4	–	–	–	–	2	–	1	1‡	–	4	–	–	–	–
9	1	–	–	–	–	1	–	–	–	–	1	–	–	–	–
13	1	2	–	–	–	3	–	–	–	–	3	–	–	–	–
18	3	–	–	–	–	3	1	–	–	–	3	–	–	–	–
24	3	1	–	–	–	–	1	3	–	–	2	1	1	–	–
31	2	1	4	1	–	1	2	1	2	2	5	3	–	–	–
10-day course															
2	2	1	–	–	–	3	–	–	–	–	2	1	–	–	–
4	3	–	–	–	–	2	1	–	–	–	2	1	–	–	–
6	3	–	–	–	–	3	–	–	–	–	2	–	1	–	–
9	3	–	1	–	–	3	1	–	–	–	4	–	–	–	–
13	3	2	–	–	–	2	2	1	–	–	4	1	–	–	–
18	–	1	3	1	–	–	3	1	–	–	4	1	–	–	–
21	2	1	–	1	1	–	2	1	–	2	4	–	1	–	–
24	–	–	–	1	2	1	–	–	1	1	4	–	–	–	–
29§	–	–	–	–	1	–	–	–	–	1	1	–	–	–	–

*Nausea and vomiting: 0 = none; 1 = mild; 2 = moderate; 3 = severe; 4 = life-threatening.
†Tumor in bone marrow.
‡Death from disseminated intravascular clotting.
§Patient inadvertently received too much drug but survived.

TABLE 3.—Days of nadir and ranges of DAG myelotoxicity

	Drug schedule	Nadir (Day)		Recovery (Day)	
		Median	Range	Median	Range
Leukopenia	5-day	15	5–36	10	3–31
Thrombocytopenia		19	5–29	18	7–42
Leukopenia	10-day	22	8–43	13	3–54
Thrombocytopenia		26	8–36	12	4–33

for at least 4 weeks; *partial response (PR)*—50% or greater decrease in the sum of the products of the greatest diameters of the measurable lesions and no appearance of new lesions for at least 30 days; *stable disease*—lesions decreased in size but <50%, were unchanged in size, or enlarged to <25%; *increasing disease*—increase of at least 25% of measured lesions or appearance of new lesions.

RESULTS

Toxicity

Myelosuppression was dose limiting for both the 5- and 10-day schedules. As shown in table 2, DAG given in daily injections for 5 days tended to produce more frequent and severe thrombocytopenia than leukopenia. Grade 2 thrombocytopenia started at the 24-mg/m² dose and reached grade 4 at 31 mg/m². At these doses about half of the patients experienced mild nausea. The maximum tolerated dose for this schedule is 30 mg/m²/day × 5 days. The 10-day schedule produced leukopenia and thrombocytopenia of equal frequency and severity with a maximum tolerated dose of 21 mg/m²/day as shown in table 2. No consistent nausea was present at higher dosages. Prior nitrosourea therapy predisposed to more myelosuppression on both schedules and the 24-mg/m² course was only carried out in patients without prior nitrosoureas. The median and range of time to the development of and recovery from myelotoxicity are shown in table 3. Of interest are the rather wide ranges which should provoke caution in future studies. There was no evidence of drug-induced hepatic, renal, respiratory, cardiovascular, or neurologic toxic effects. Extravasation of DAG induces severe local erythema; in one patient, it produced a 4 × 9-cm eschar which healed slowly.

Responses

Two responses were noted: one PR of 3 months' duration (laryngeal carcinoma with extensive prior chemotherapy) and one CR of 9 months' duration (adenocarcinoma of the lung with prior radiation therapy). Both responses were achieved at doses producing no significant toxicity on the 10-day schedule. The long CR was maintained with monthly 10-day courses of 9 mg/m² with resultant mild-to-moderate myelosuppression. One patient with epidermoid lung cancer had demonstrable tumor shrinkage after each of two courses of DAG, but regrowth occurred in each case; the net reduction was inadequate to define as a PR. There were five patients with stable disease and one with PR among 12 patients with squamous cell carcinomas, while we observed three patients with stable disease and one with CR among 16 patients with adenocarcinoma. We studied a total of 37 patients who were evaluable for response; 26 had increasing disease, most of whom received submaximal doses.

DISCUSSION

DAG was subjectively well tolerated by all patients. Myelosuppression was dose limiting. For phase II studies, the recommended starting doses are 30 mg/m²/day × 5 days or 20 mg/m²/day × 10 days for patients with a good bone marrow reserve, renal and hepatic function, and no prior nitrosourea therapy. These doses should be reduced to 25 mg/m²/day × 5 days or 15 mg/m²/day × 10 days if previous nitrosourea therapy has been used. In addition, it should be noted that only patients with normal hepatic and renal function were studied. Investigators would do well to be extra cautious in patients with these impaired functions.

Pharmacokinetic studies in man are needed to establish the route(s) of excretion and/or degradation. Institóris et al studied DAG labeled with tritium at position 1 and injected into rats and mice (9). Sixty-seven percent of the label appeared in the rats' urine within 6 hours, while 39% of the label appeared in the urine of mice in 8 hours. In neither group was the proportion of active drug/metabolite(s) determined. Renal excretion may thus be of importance in man. Further experience may empirically define the importance of these variables. The responses described are encouraging and the Southwest Oncology Group has initiated phase II studies of DAG.

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Competitive Dihydrofolate Reductase Binding Assay of Triazinate (NSC-139105), Methasquin (NSC-122870), and Aminopterin (NSC-739)[1,2]

Brief Reports and Preliminary Communications

Charles E. Myers,* Helen M. Eliot, and Bruce A. Chabner[3]

In an effort to obtain antifolates with less host toxicity and better therapeutic efficacy than methotrexate (MTX),[4] a large number of alternate inhibitors of dihydrofolate reductase have been synthesized (1). Several of these compounds differ sufficiently from MTX in various pharmacologic properties to be of clinical interest (2,3) and are currently being used in clinical trial. However, experience with MTX in intrathecal-therapy regimens for the treatment of leukemic meningitis (4) and in high-dose regimens for the treatment of osteogenic sarcoma (5) has indicated that delayed drug elimination may play an important role in MTX toxicity. For this reason, clinical trials involving other antifolate compounds may also benefit from routine pharmacokinetic monitoring. Unfortunately, determination of antifolates, including MTX, in clinical samples has not been possible in most institutions due to the complexity of existing assay methods. Recently, this and several other laboratories have described competitive protein-binding assays for MTX based on the affinity of this drug for dihydrofolate reductase; these methods are highly specific, rapid, and sensitive (6–8). This report describes the modification of one of these recently developed assays for MTX to allow determination of several other folate analogs in clinical samples.

[1]Received Oct 17, 1975; revised Feb 24, 1976; accepted Mar 8, 1976.

[2]Triazinate: CAS reg. No. 41191-04-2; ethanesulfonic acid, compd. with α-[2-chloro-4-(4,6-diamino-2,2-dimethyl-*s*-triazin-1(2*H*)-yl)phenoxy]-*N*,*N*-dimethyl-*m*-toluamide (1:1).

Methasquin: aspartic acid, *N*-[*p*-[[(2,4-diamino-5-methyl-6-quinazoliinyl)methyl]amino]benzoyl]-, hydrate (5:3), L-.

Aminopterin: CAS reg. No. 54-62-6; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl]methyl]amino)benzoyl]-.

All drugs were supplied by Drug Research and Development Program, Division of Cancer Treatment (DCT), National Cancer Institute (NCI).

[3]Medicine Branch, Clinical Oncology Program (COP) (Drs. Myers and Eliot) and Laboratory of Chemical Pharmacology, Experimental Therapeutics Program (Dr. Chabner), DCT, NCI, Bethesda, Md.

[4]Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

**Reprint requests to:* Charles E. Myers, MD, Medicine Branch, COP, DCT, NCI, Bldg 10, Rm 12N226, National Institutes of Health, Bethesda, Md 20014.

MATERIALS AND METHODS

Partially purified dihydrofolate reductase from dichloromethotrexate-resistant *Lactobacillus casei* (*L. casei*) was obtained from the New England Enzyme Center, Boston, Mass. This enzyme preparation formed 4.6 μmols of tetrahydrofolate/minute/mg of protein at pH 7.5 and 37°C and was able to bind 1.9 nmols of MTX/mg of protein at pH 6.2 and 23°C. The dihydrofolate reductase from MTX-resistant L1210 leukemia cells has been previously characterized (2). Unlabeled MTX purified by chromatography on diethylaminoethyl cellulose as previously described (9) was a gift of Dr. David G. Johns, NCI. 3′,5′,9(*n*)-^{3}H-MTX (9.5 curies/mmol) was obtained from Amersham Searle, Arlington Heights, Ill. Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), Norit A, a high molecular weight dextran (average molecular wt 200,000–275,000), and bovine serum albumin, Fraction V, were all obtained from the Sigma Chemical Co, St. Louis, Mo.

The assay is based on competition between ^{3}H-MTX and unlabeled drug for binding to dihydrofolate reductase, with subsequent removal of unbound drug by charcoal adsorption (6). The assay was performed at 23°C and involved rapid sequential addition of the following: (*a*) 0.58 pmol (4.95 nanocuries of ^{3}H-MTX and 50 units of heparin in 150 μl of 0.1 M potassium buffer, pH 6.2; (*b*) 200 μl of a serially diluted patient sample adjusted to pH 6.2 with 1 N HCl or standard solution of unlabeled drug; and (*c*) 100 μl of a 3.5 × 10^3-fold dilution of the stock enzyme preparation of 0.5 M potassium phosphate buffer, pH 6.2, containing 0.5 pmol of MTX binding activity. This third solution also contained 0.24 μmol of freshly prepared NADPH.

Immediately after addition of the enzyme-NADPH solution, the tubes were mixed by Vortex agitation and 25 μl of a charcoal slurry was added (Norit A, 10 g/100 ml; bovine serum albumin, Fraction V, 2.5 g/100 ml; and high molecular weight dextran, 0.1 g/100 ml, adjusted to pH 6.2 with 1 N HCl). The samples were mixed by Vortex agitation and centrifuged at 700 *g* for 30 minutes. A 200-μl aliquot of the supernatant was then placed in 10 ml of Aquasol and the enzyme-bound ^{3}H-MTX was

determined by counting in a liquid scintillation spectrophotometer with an efficiency of 41%.

Calculations

A standard curve for competitive ligand binding may be constructed by plotting the log of unlabeled MTX added versus the ratio of ^{3}H-MTX bound in the experimental determination (B) to the ^{3}H-MTX bound in the control assay (B_0). Transformation of the ordinate to logit form

$$\text{logit} = \ln \frac{B/B_0}{1 - (B/B_0)}$$

yields a linear standard curve. To facilitate calculation of clinical results, a Wang 600 table-top calculator was programmed to perform the following in sequence: (*a*) logit transformation of standard and patient data, (*b*) determination of the standard curve by unweighted least squares regression analysis, and (*c*) calculation of individual patient values from the resulting linear regression equation.

RESULTS

The four antifolates tested showed a wide range of affinities for *L. casei* dihydrofolate reductase with 50% displacement values ranging from 1×10^{-8} M for MTX to 1×10^{-5} M for triazinate (table 1). While this assay appears to have adequate sensitivity for MTX, aminopterin, and methasquin, it is of little value in the assay of the triazinate. Because dihydrofolate reductase from MTX-resistant L1210 leukemia cells is known to possess a high affinity for the triazinate (2), the enzyme from this source was substituted for the *L. casei* enzyme in the competitive binding assay. With this binding protein the 50% displacement value for the triazinate decreased to 1×10^{-7} M and the 5% displacement value to 1×10^{-8} M. Parallel competition studies done with each drug dissolved in undiluted plasma indicated drug recoveries were uniformly > 90% whether the L1210 cells or the *L. casei* enzyme was used as binding proteins. Both the L1210 cells and the *L. casei* enzyme yielded linear logit versus log (drug) standard curves with a useful range extending from one log below to one log above the 50% displacement value.

TABLE 1.—Competition between labeled MTX and various antifolates for *L. casei* dihydrofolate reductase

Drug	50% displacement	5% displacement*
MTX	1×10^{-8} M	1.5×10^{-9} M
Aminopterin	5×10^{-8} M	5×10^{-9} M
Methasquin	5.4×10^{-8} M	5×10^{-8} M
Triazinate	1×10^{-5} M	1×10^{-6} M

*Lower limit of sensitivity of the assay.

DISCUSSION

The recent development of competitive protein-binding assays for MTX promises to make routine pharmacokinetic monitoring of MTX practical (6–8). Although these initial reports speculate on the possible application of this assay technique to the measurement of other antifolates, direct evidence for this has been lacking. The present study has shown that one of these assay procedures (6), based upon the commercially available *L. casei* dihydrofolate reductase, may be used to measure aminopterin and methasquin, but not triazinate.

While high affinity for MTX appears to be a characteristic of dihydrofolate reductase from most sources, affinity of the enzyme for other antifolates differs significantly from species to species (10). It is possible that through the use of an appropriate enzyme source, the competitive binding procedure outlined in this paper may be modified to allow convenient measurement of a wide variety of antifolates in biologic fluids. An example of this is the use of the L1210 enzyme to measure the triazinate as shown in this study. In a similar way it is likely that the relative affinities of the various antifolates for human dihydrofolate reductase can be determined with considerable accuracy.

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Liposomal Potentiation of the Antitumor Activity of Alkylating Drugs[1,2]

Brief Reports and Preliminary Communications

R. J. Rutman,* C. A. Ritter, N. G. Avadhani, and J. Hansel[3]

Numerous studies have demonstrated that artificial liposome vesicles (1,2) can enter animal cells without toxicity (3) and can transport drugs and other molecules to intracellular sites (4–10). Liposome-mediated transport can produce an altered pattern of retention and distribution of the transported materials (5). Based on these properties, Gregoriadis and Neerunjun (7) recently observed that liposomes could enhance the antitumor activity of actinomycin D,[4] a lipid-soluble drug. We now wish to report evidence of liposomal activation of two highly reactive and unstable alkylating agents, BCNU and mechlorethamine (HN2),[5] against two mouse tumors, Ehrlich ascites and L1210 leukemia.

MATERIALS AND METHODS

Ehrlich ascites tumors were grown in Swiss mice using an inoculum of 10^7 cells. L1210 lymphocytic leukemia was passed in DBA/2 mice; for test, 2.5×10^6 cells were transplanted to CDF_1 hybrids. Variable drug schedules were used as indicated in table 1.

Liposome drug combinations were prepared by the direct admixture of sonicates of dipalmitoylphosphatidylcholine (L-α-lecithin) (Sigma Chemical Co, St. Louis, Mo) with equimolar amounts of drug. The sonicates were prepared by exposing a lecithin suspension in 0.9% sodium chloride to 10–20 seconds of sonication at setting 1, 2.5 amperes, in a Branson 3125 sonifier. The suspensions were stable, and were injected in volumes of 0.2 ml/dose. No effort was made to separate liposomes from unbound drugs.

RESULTS

Table 1 summarizes the effect of liposome drug mixtures with either HN2 or BCNU on both the Ehrlich ascites tumor and the L1210 lymphocytic leukemia. It can be seen that the effects of HN2 on the Ehrlich ascites tumor were increased when either a single- or a multiple-dose protocol was used, and that the effects of the multiple-dose regimen were quite significant. Similar results with the Ehrlich ascites tumor were also obtained in the single-dose experiment with BCNU.

With the L1210 ascites tumor, the dose of 15 mg/kg for 2–6 days had only a small effect on mean survival time; in combination with the liposome carrier, this dose approached the curative level predictable with BCNU in this tumor. The effectiveness of the higher dose of 30 mg/kg was neither enhanced nor diminished by incorporation into phospholipid vesicles.

Appropriate controls have shown that L-α-lecithin sonicates by themselves have no effect on the survival of tumor-bearing mice and that cells pretreated with liposomes in vivo are not sensitized to the drugs.

DISCUSSION

Our observations that liposomes have the ability to potentiate antitumor action confirm and extend those of Gregoriadis and Neerunjun (7). Our results also provide additional evidence for the thesis that these phospholipid vesicles can be used to alter the distribution and activity of drugs. It is of interest to indicate the particular rationale behind our experiments. During mechanistic studies of HN2, we found that mitochondria in situ undergo significant inhibitions of RNA and protein synthesis (11), as well as specific interferences in respiration[6] at low doses of HN2. In contrast to this, isolated purified mitochondria are highly resistant to HN2; doses 250–500 times greater than those which inhibit the organelles in vivo have no effects in vitro. As a possible explanation of the intracellular versus extracellular difference, we reasoned that the intracellular-antimitochondrial action of HN2 might be

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[2]Supported in part by Public Health Service grant CA-05295 from the National Cancer Institute (NCI), National Institutes of Health (NIH), Department of Health, Education, and Welfare.

[3]Department of Animal Biology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia.

[4]Actinomycin D: NSC-3053.

[5]BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-. Supplied by NCI, NIH, Bethesda, Md.

Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2′-dichloro-*N*-methyl-, hydrochloride. Supplied by Merck Sharp and Dohme, Rahway, NJ.

**Reprint requests to:* Dr. R. J. Rutman, Department of Animal Biology, School of Veterinary Medicine, University of Pennsylvania, 3800 Spruce St, Philadelphia, Pa 19104.

[6]Ritter CA, and Rutman RJ. Unpublished results.

TABLE 1.—Potentiating effects of liposomal carriage on antitumor activity of HN2 and BCNU

Tumor type*	Drug†	Dose (mg/kg)/ schedule (days)	Mean survival time (± SD)	% survivors 60 days	% survivors 120 days
Ehrlich ascites	—	—	16 ± 3.0	—	—
Ehrlich ascites	HN2	0.4/2–6	29	0	0
Ehrlich ascites	HN2-L‡	0.4/2–6	>75	50	30
Ehrlich ascites	HN2	2/6	47	0	0
Ehrlich ascites	HN2-L	2/6	57	0	0
Ehrlich ascites	BCNU	30/6	40	20	0
Ehrlich ascites	BCNU-L	30/6	>70	60	30
				% survivors	
				3 wks	*6 wks*
L1210 leukemia	—	—	7.0 ± 0.7	—	—
L1210 leukemia	BCNU	15/2–6	19	0	0
L1210 leukemia	BCNU-L	15/2–6	>40	50	33
L1210 leukemia	BCNU	30/2–6	>30	83	50
L1210 leukemia	BCNU-L	30/2–6	>33	83	50

*10^7 Ehrlich cells inoculated ip into 20 control and 10 experimental animals; 2.5×10^6 L1210 cells inoculated ip into 20 control and 6 experimental animals.

†Administered ip.

‡L = equimolar mixture of drug and liposomal preparation (mols drug/mols phospholipid).

mediated by a hydrophobic, chemically unreactive carrier. Accordingly, we tested the effects of combinations of HN2 and liposomes against mitochondria in vitro and found that the combination duplicated the in vivo inhibitory effects of HN2.[7]

We still have no direct evidence of the mode of carriage of either HN2 or BCNU. It is most unlikely that uptake of drug by the vesicles could facilitate transport since at equimolar concentrations of drug and phospholipid, unusually high intravesicular concentrations would need to be achieved. It is conceivable, however, that the drugs could dissolve in the bilipid membrane layers and thus bypass the permeability barrier of the cell membrane because liposomes can fuse with cell membranes as well as enter cells (3,9). This process would need to be relatively efficient since both BCNU and HN2 enter cells readily and a specific HN2 transport system has been identified (12). However, the data we have obtained with mitochondria suggest another possibility (at least for HN2), ie, that the liposome vesicle facilitates drug attack on membrane-bound biosynthetic complexes. These are known to exist in mitochondria (13). It thus becomes of some importance to establish the mode of carriage by the liposomes of drugs such as HN2 and BCNU.

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Chemotherapy of Metastatic Prostate Adenocarcinomas in Germfree Rats. I. Effects of Cyclophosphamide (NSC-26271)[1,2,3]

Brief Reports and Preliminary Communications

Morris Pollard* and Phyllis H. Luckert[4]

Early reports on cancer metastasis describe the spread of neoplastic cells to distant sites through blood and/or lymph channels and by direct extension through body cavities (1,2). There is need for a model tumor system in which the pattern of metastasis is uniform, predictable, and does not require special manipulations. The transplantable rat prostate adenocarcinoma described in this paper satisfies these requirements and has been applied in therapeutic trials with cyclophosphamide (CPA).

Metastasizing adenocarcinomas appeared spontaneously in the prostate glands of aged germfree (GF) Lobund Wistar rats (3). Three metastasizing tumor lines (I, II, and III) were transplanted from them to male and female GF and conventional Wistar rats. The tumors spread from the sc implant site through ipsilateral regional lymph nodes to the lungs of all Lobund Wistar rats (4).

It had been determined that GF mice and rats tolerated larger subtoxic doses of CPA over longer periods than conventional counterpart animals (5,6) because the latter developed complicating infections. GF and conventional rats, with metastasizing prostate carcinoma cells, were subjected to treatment schedules of CPA which modified the pattern of established local and metastatic lesions.

METHODS

Male and female weanling conventional and GF Lobund Wistar rats were inoculated sc in the dorsolumbar region with cells derived from minced prostate tumors (type I) which were being transplanted serially (4). Small local tumor nodules appeared within 10 days and ulcerated within the next 10 days. In the meantime, expanding foci of tumor cells appeared in draining axillary lymph nodes which were eventually replaced by tumor cells. Distinct expanding round tumor foci appeared on and in the lungs which were usually very numerous by Day 50 after inoculation.

At various intervals after inoculation of tumor cells, GF and conventional rats were injected ip with CPA freshly dissolved in water. It had been determined that conventional weanling rats given 5 mg of CPA (30 mg/kg body wt) once per week developed bacterial infections; however, GF counterpart rats survived indefinitely on the same treatment schedule with no evidence of disease. The start of weekly CPA treatments ranged from 1 to 28 days after inoculation of tumor cells and usually extended for periods beyond the survival limits of the untreated tumor-bearing rats. At intervals after drug treatment was discontinued, the rats were killed and examined for gross and microscopic distribution of tumor lesions.

For final examinations each rat was examined for body weight, white blood cell count, and hematocrit level and was then anesthetized with ether and exsanguinated from the heart. Tumors and enlarged lymph nodes were weighed and portions thereof, plus other visceral organs, were fixed in Bouin's solution and further processed for histologic examinations. The intact lungs were inflated with Bouin's solution by a syringe through the trachea and 24 hours later they were immersed in 70% ethanol. The number of white surface tumors on a yellow background were then counted with the aid of a duoloupe, and the lungs were then processed for histologic examinations.

GF rats were maintained in plastic isolators on sterile diet L485 and water ad libitum. They were negative for bacteria on the basis of weekly examinations. The same protocol was applied to conventional rats maintained on the same sterilized diet in laminar air flow units or in an open clean animal room.

RESULTS

As indicated in table 1 (experiment No. 1), GF rats

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[3]Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate. Supplied by Mead-Johnson Co, Evansville, Ind.

[4]Lobund Laboratory, The University of Notre Dame, Ind.

**Reprint requests to*: Dr. Morris Pollard, Lobund Laboratory, The University of Notre Dame, Notre Dame, Ind 46556.

TABLE 1.—CPA in GF rats with prostate carcinoma type I*

Experiment No.	No. of rats	CPA treatment	No. of days— To treatment	Of treatment	Without treatment	From start of study	Average tumor wt/g (range)	Average No. of lung tumors (range)
1	4	–		—	56	56	7.95 (3–15)	469 (204–671)
	8	+	7	42	7	56	0.12 (0–27)†	0
2	4	–			71	71	6.8 (7.7–10.1)	184 (47–475)
	2	+	1	70	0	71	0	0
	2	+	1	70	30	101	0	3 (2–4)
	2	+	14	56	0	70	0	0
	2	+	14	56	30	101	0.4 (0–0.83)†	3 (1–5)
3	2	–			47	47	5.9 (5.7–6.1)	345 (305–385)
	7	+	28	42	0	70	1.3 (0.7–1.8)	14 (4–41)

*Tumor cells inoculated sc in the dorsolumbar area. CPA treatments (5 mg/wk ip) were started at specified days after inoculation of tumor cells, and rats were killed at specified days after end of treatment schedule.
†Sclerosed tissue on histologic examinations.

were treated with CPA 7 days after tumor inoculation and at weekly intervals for 6 weeks. They were in excellent physical condition when killed 1 week after the last treatment (56 days after inoculation of tumor cells), and they carried only sclerosed remnants of sc tumors. The untreated GF rats had large ulcerated sc tumors (average 7.9 g), swollen axillary lymph nodes, and numerous tumor foci in the lungs (average 469/rat).

Application of the same treatment regimen in conventional rats maintained in the laminar air flow units resulted in tumor-free subjects, although with minimal evidence of pneumonia. The untreated control rats had extensive sc tumors, large axillary lymph nodes, and an average of 264 lung tumors per rat. In a parallel trial conducted in an open animal room, the CPA-treated rats had extensive pneumonia and abscesses in active primary tumors and in the tumor-bearing lymph nodes. Because of this it was difficult to assess their tumor weights and numbers of lung tumors.

In a second trial (table 1, experiment No. 2), two groups of GF rats were treated on Day 1 or 14 after inoculation of tumor cells. Each group of rats was treated with CPA for 8 or 10 weeks and then each rat was examined 1 or 30 days after the last drug treatment. Although tumors were not detected 1 day after the last treatment, pinpoint lung tumors (average 3/rat) were detected in all rats 30 days after the last CPA treatment (100 days after inoculation of tumor cells). The CPA-treated rats appeared healthy; however, the untreated rats were very sick 63 days after inoculation of tumor cells. They had large sc tumors (average 6.8 g), large axillary lymph nodes, and numerous distinct and coalesced lung metastases (average 184/rat).

In a third trial (table 1, experiment No. 3), rats were treated with CPA on Day 28 after sc inoculation of prostate tumor cells. Examination of one untreated rat on Day 28 revealed a distinct local tumor, swollen axillary lymph node, and 50 lung tumors. On Day 70 (42 days after CPA treatment was started), the treated rats appeared healthy and had small sc tumors (average 1.3 g) and a few indistinct lung lesions (average 14/rat). The tumor tissues in the CPA-treated rats appeared sclerosed, but occasional tumor cells were observed sequestered among the amorphous fibers. On Day 47, the untreated rats had large sc tumors (average 5.9 g), swollen axillary lymph nodes, and active lung metastases (average 345/rat).

DISCUSSION

The prostate tumor system is very sensitive to the cytotoxic effects of CPA given once per week. Early treatments with CPA prevented the appearance and subsequent metastatic spread of the tumor. When the start of therapy was delayed, CPA treatments not only interrupted continued tumor growth but actually destroyed most (but not all) of the primary and metastatic lesions. In both early and late treatment schedules, the CPA-treated GF rats appeared healthy: their lymph nodes, spleens, and thymus glands resembled the organs of untreated GF rats.

Although the benefits of the CPA regimen were clearly defined in the GF rats, many of the unprotected conventional counterpart rats were sick with combinations of actively growing tumors and abscesses. It would be important to determine if bacterial complications actually interfere with the carcinostatic effects of CPA.

Treated GF rats initially appeared "cured"; how-

ever, inapparent foci of tumor cells in the lungs were activated after CPA treatments were discontinued. Thus, protocols for assessment of treatment regimens should require long-term observations, especially after treatment is discontinued.

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Phase II Trial of 5-Fluorouracil (NSC-19893), Adriamycin (NSC-123127), and Methotrexate (NSC-740) in Lung Adenocarcinoma[1,2]

Barry B. Lowitz[3,*]

Lung adenocarcinoma has been generally refractory to chemotherapeutic attack. In the present study the choice of drugs was based on reports of their activity as single agents. Methotrexate is reported to produce a 32% response rate in lung adenocarcinoma (1). Adriamycin has a response rate of up to 25% (2,3). There is some evidence that cells in tissue culture have reciprocal sensitivity to adriamycin and methotrexate; cells resistant to one of these agents may be killed by the other (4). The use of the two agents in combination for clinical trials is, therefore, of interest.

When the present study began, 5-fluorouracil (5-FU) was undergoing clinical testing for lung adenocarcinoma. There had been one report of a 40% response rate in a small group of patients (5). Also, Bonadonna et al had reported five responses among six patients with lung adenocarcinoma who received a combination of 5-FU with procarbazine, but no responses in five patients treated with procarbazine alone (6). These studies suggested activity for 5-FU.

In subsequent studies 5-FU alone has shown little activity. However, 5-FU and methotrexate inhibit the formation of thymine by different pathways; while synergy between these two agents has not been shown, the inclusion of 5-FU in a combined-drug regimen appeared worthy of trial.

Patients and Methods

Eleven patients with biopsy-proven metastatic adenocarcinoma of the lung were treated with a three-drug iv regimen: adriamycin (40 mg/m^2 q28 days), 5-FU (300 mg/m^2 weekly), and methotrexate (25 mg/m^2 weekly). Doses were omitted when there was grade II or greater stomatitis, or when the white blood cell (wbc) count was <3500 cells/mm^3. All patients had creatinine clearance >60 ml/minute. For serum bilirubin levels >2 mg/100 ml, the adriamycin dose was decreased by 50%.

All patients were given a performance status (PS) rating on a scale of 1–10 (7) prior to therapy. The PS was determined on the following basis:

1. Moribund; estimated survival <1 week.
2. Critically ill. Extensive acute nursing requirements making hospitalization mandatory; anticipated survival <2 weeks.
3. Major nursing needs which cannot be handled at home; patient bedridden but able to feed self.
4. Same as 3, but patient can be managed at home with help.
5. Same as 4, but patient able to walk short distances.
6. Confined to the home but takes care of most personal needs.
7. Can leave home on occasion for walks or visits with friends.
8. Can conduct most normal activities and/or works on a part-time basis.
9. Symptoms or signs of cancer are present but do not interfere with full-time work and activities.
10. Same as 9, without symptoms of disease.

Response categories included: (*a*) CR—no evidence of cancer; (*b*) PR—a 50% decrease in the sum of the products of the diameter of all measurable disease lasting at least 1 month with no new disease appearing; (*c*) stabilization (S)—same as PR but <50% decrease; and (*d*) progression (NR)—all patients not falling into the other categories and those who died before completing the first course of therapy. All patients were considered evaluable, and all were consecutively entered and were not selected by criteria other than the diagnosis of metastatic lung adenocarcinoma.

Of the 11 patients there were one CR, four PRs, three Ss, and three NRs. Responses by site are shown in table 1. All nonresponding patients had an initial PS of 2 or less and none lived >2 weeks after therapy started. All eight patients with an initial PS of 3 or greater stabilized or responded.

Of patients previously treated, one of one patient

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[2]5-Fluorouracil: CAS reg. No. 51-21-8.

Adriamycin: CAS reg. No. 23214-92-8.

Methotrexate: CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

[3]Investigative Therapeutics Branch, Hematology-Oncology Division, Department of Medicine, Veterans Administration (VA) Hospital, Sepulveda, Calif.

**Reprint requests to:* Barry B. Lowitz, MD, Investigative Therapeutics Branch, Hematology-Oncology Division, VA Hospital, 16111 Plummer St, Sepulveda, Calif 91343.

TABLE 1.—Responses to 5-FU, adriamycin, and methotrexate by site

Site	CR	PR	S	NR
Nodular lung	1	1	1	2
Lymphangitic lung	0	0	0	2
Skin	0	2	0	0
Bone	0	1	1	0
Bone marrow	0	0	0	1
Pleural effusion	0	0	2	1
Liver	1	1	1	2
Vena cava obstruction	1	0	0	0

with previously irradiated skin metastases responded over the irradiated site; one patient who failed to respond to cyclophosphamide and methotrexate responded; one patient treated with cyclophosphamide and CCNU failed to respond; and one patient who progressed with 5-FU and BCNU stabilized. One patient who recurred with superior vena cava syndrome following radiation therapy obtained complete resolution of his symptoms.

The one patient whose response lasted only 1 month required a 50% dose reduction because of stomatitis and subsequently progressed on the lower-dose regimen. All other responses, to date, have lasted from 8 to 32 weeks. Until now, the only patient who has progressed did so after 26 weeks of stable disease (the initial PS was 4).

When stratified by PS and extent of disease, all responding or stable patients have survivals exceeding the median of a large historical untreated control group (7). All stable or responding patients showed stabilization or responses by the end of the first 4 weeks of therapy. Patients surviving long enough to receive therapy for >1 month rarely missed a dose of the weekly 5-FU and methotrexate. Missed doses resulted from patient wishes in two cases and intercurrent infection in two. Except for one patient who developed moderate stomatitis and a wbc count nadir of 1000 cells/mm^3, all responding or stabilizing patients were able to receive the planned courses of therapy. It was possible to increase the 5-FU and methotrexate by 20% in three patients without producing a wbc count of <3500 cells/mm^3 or stomatitis.

PS changes with therapy were variable. The two patients with initial PSs of 8 and 9 initially lost weight but subsequently gained more than they had lost and had appetite improvement. Both patients had relief of local symptoms of their disease. One patient with a PS of 4 and painful bony metastasis had pain relief with a decrease in nursing care needs, but subsequently lost weight. One patient with pleural disease had a decrease in dyspnea. The remaining patients did not show improvement in their PS: these included one patient (PS 4) who had a CR in liver metastases and a lung nodule, another patient (PS 6) with painful but responding skin metastases, and a patient who stabilized with widespread disease. Of these, the second patient did not attain the target doses of drugs.

The toxic effects consisted of stomatitis (one of 11 patients), nausea (two of 11), wbc count <1500 cells/mm^3 (two of 11), infection (one of 11), malaise occurring only during the first 4 days of the course (three of 11), and alopecia (four of 11). Except for one patient with bone marrow metastases and initial thrombocytopenia, no patient had platelet counts <100,000 cells/mm^3, vomiting, skin changes, or palpitations. Fatal infection occurred in one patient (initial PS 2) who developed pneumonia and died 2 weeks after therapy was started when his wbc count was 900 cells/mm^3. Two patients developed septic pleural effusions during chest tube drainage after the first course of therapy; these episodes were not related to leukopenia and the patients recovered without sequellae.

To date, 5-FU, adriamycin, and methotrexate therapy appears to be an effective means of temporarily controlling metastases and prolonging survival in lung adenocarcinoma with little incapacitation from drug toxicity. Improvement in the PS was variable, but was noted most often in those patients with initially high ratings. Responding or stable patients with low PSs tended to remain in their initial performance category despite their apparently improved survival time. The regimen has proved to be of no value in our patients with a PS <2. This may be related to insufficient time for the drugs to take effect in the presence of rapidly advancing cancer. Both stabilization and response are associated with improved survival over historical controls.

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Mechlorethamine (NSC-762) Plus CCNU (NSC-79037) in the Treatment of Inoperable Squamous and Large Cell Carcinoma of the Lung[1,2]

Brief Reports and Preliminary Communications

John H. Edmonson,* Stephen Lagakos, Leo Stolbach, Charles P. Perlia, John M. Bennett, Edward G. Mansour, John Horton, William Regelson, F. J. Cummings, Lucien Israel, Isadore Brodsky, Bruce I. Shnider, Richard Creech, and Paul P. Carbone[3]

This study was developed in 1971 to determine whether combination treatment with CCNU and mechlorethamine would be therapeutically superior to mechlorethamine alone in patients with inoperable squamous and large cell carcinoma. The idea for combining CCNU with this standard alkylating agent was based on evidence in Lewis lung tumor in mice that methyl-CCNU combined with cyclophosphamide produced better results than the maximal responses to the two component drugs used alone (1). We used mechlorethamine instead of cyclophosphamide because of its reported superiority as a single agent against squamous cell carcinoma (2). CCNU was selected as the nitrosourea for this combination regimen because of its availability for use in comparative clinical studies at that time.

MATERIALS AND METHODS

Patients with inoperable squamous cell and large cell carcinoma were accepted in our study if they had had histologic confirmation, if they had not previously received cytotoxic drug treatment, and if they had adequate renal and bone marrow function (blood urea nitrogen $\leq$ 20 mg/100 ml or creatinine $\leq$ 1.5 mg/100 ml; leukocytes $\geq$ 5000 cells/μl, and platelets $\geq$ 100,000 platelets/μl). Previous surgery or radiation therapy was not a basis for patient exclusion; however, if patients had had prior radiation therapy it must have been completed > 1 month before this study and disease progression was required.

The ECOG Operations Office centrally randomized 163 patients with squamous cell and 92 patients with large cell carcinoma of the lung to receive either mechlorethamine (15 mg/m^2) alone every 3 weeks iv or mechlorethamine (10 mg/m^2) every 3 weeks iv plus CCNU (70 mg/m^2) every 6 weeks orally. Treatment was continued until objective evidence of disease progression was present in each case. Brain metastases appearing during treatment were to be treated with cranial radiation or glucocorticoids and chemotherapy was continued until other signs of progression were noted. Those patients who received mechlorethamine alone during the primary treatment trial were scheduled to begin treatment with CCNU (130 mg/m^2) orally every 6 weeks after failure with mechlorethamine. Dosage modifications were specified according to the degree of myelosuppression present at each treatment visit.

Patients were evaluated for objective tumor changes and were classified as having: *complete*

[1]Received Nov 14, 1975; revised Mar 19, 1976; accepted Mar 25, 1976.

[2]Mechlorethamine: CAS reg. No. 55–86–7; diethylamine, 2,2′-dichloro-*N*-methyl-, hydrochloride.

CCNU: CAS reg. No. 13010–47–4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-.

[3]This study was done by the Eastern Cooperative Oncology Group (ECOG) (Paul P. Carbone, chairman; CM-33745) and was supported by Public Health Service grants from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare. The following institutions participated in the study: Maimonides Medical Center, Brooklyn, NY (*J. H. Edmonson*; CA-05588); Statistical Center, State University of New York, Buffalo (*S. Lagakos*; CA-12721); Tufts University School of Medicine, Boston, Mass (*L. Stolbach*; CA-07190); Rush-Presbyterian-St. Luke's Medical Center, Chicago, Ill (*C. P. Perlia*; CA-10948); University of Rochester School of Medicine, NY (*J. M. Bennett*; CA-11083); Case Western Reserve University, Cleveland, Ohio (*E. G. Mansour*; CA-14548); Albany Medical College of Union University, NY (*J. Horton*; CA-06594); Medical College of Virginia, Richmond (*W. Regelson*; CA-10572); Brown University, Providence, RI (*F. J. Cummings*; CA-15947); Centre Hospitalier Universitaire Lariboisier, Paris, France (*L. Israel*); Hahnemann Medical College, Philadelphia, Pa (*I. Brodsky*; CA-13611); Georgetown University School of Medicine, Washington, DC (*B. I. Shnider*; CA-02824); and American Oncologic Hospital, Philadelphia, Pa (*R. Creech*). Other participating institutions were: University of Alberta (CA-13314); Albert Einstein College of Yeshiva University (CA-14958); Boston University Medical Center (CA-11109); University of Iowa; Jackson Memorial Hospital (CA-02822); Jefferson Medical College (CA-14215); Lahey Clinic Foundation (CA-12880); University of Manitoba; Mt. Sinai Hospital Medical Center (CA-14144); Mt. Sinai Medical Center; University of Ottawa; Pennsylvania Hospital (CA-13613); University of Pennsylvania Hospital (CA-15488); and Yale University School of Medicine (CA-15485).

*Reprint requests to: John H. Edmonson, MD, Division of Medical Oncology, Mayo Clinic, Rochester, Minn 55901.

regression, complete disappearance of all clinically detectable cancer; *partial regression*, a decrease by at least 50% in the product of the longest perpendicular diameters without progression of any lesion or the appearance of any new lesion (for evaluable but nonmeasurable disease, partial objective regression was indicated by a definite decrease in the size of disease areas amounting to an estimated 80% or better regression [3]); *disease progression*, an increase of at least 50% in the product of the longest perpendicular diameters of measurable lesions, the appearance of any new lesion, or a definite increase in lesions which were evaluable but nonmeasurable; and *no change or stability of disease*, the lack of objective progression of disease during the first 3 or more months of treatment in the absence of objective regression.

RESULTS

Of the 255 cases included in this trial, final patient records were not received for six patients (four squamous, two large cell); 14 other patient records were judged to be unevaluable and were excluded from the analysis. Thus, 148 squamous and 87 large cell carcinoma patients were available for analysis.

Drug Toxicity

Complications due in some degree to treatment were predominantly vomiting and myelosuppression. Four of the five identified occurrences of lethal toxicity were myelosuppressive; the fifth patient who died as a result of treatment did so shortly after a severe episode of vomiting. This was classified as a drug-related death although cardiac metastases were found at autopsy.

Overall, 35% of the 117 patients who received mechlorethamine alone experienced severe, life-threatening, or lethal toxic reactions compared with 25% of the 118 patients who received the combination regimen. It is also interesting that four of the five treatment-related deaths occurred in patients receiving the standard single-drug mechlorethamine regimen. Thus, in our patient population it seems clear that the single-drug regimen was somewhat more toxic than the combination regimen. Patients who had received previous radiation therapy experienced a significantly ($P = 0.05$) higher incidence (36% versus 25%) of severe, life-threatening, or lethal toxic reactions than those without previous radiation therapy. We considered whether more severe toxic reactions might be more common in older patients but found no evidence to support this theory.

Objective Tumor Regression

Twelve (5%) of the 235 patient records were not sufficiently complete for objective evaluation (eg, a patient who refused treatment after one dose and for whom only an initial chest roentgenogram was obtained). Table 1 indicates that objective tumor responses to the two regimens were similar in both squamous cell and large cell carcinoma. No complete tumor regression was seen with either treatment; the overall partial regression rate for the entire study was only 7%.

In order to identify any correlation between objective tumor regression and toxicity, patients evaluable for objective tumor response were cross-classified with their worst degree of drug-related toxicity. There were no objective tumor regressions among those five patients who experienced lethal toxicity; however, tumor regression occurred in 17% of those experiencing severe or life-threatening toxicity but in only 5% of the patients with moderate, mild, or no toxic effects. This correlation between response rate and more pronounced nonlethal toxicity is both statistically significant ($P = 0.01$) and consistent over treatments and cell types.

Survival

The estimated median survival times were 10.6 and 11.9 weeks, respectively, for mechlorethamine alone and the combination regimen in patients with squamous cell carcinoma. For patients with large cell carcinoma the corresponding medians were 9.7 weeks for mechlorethamine and 11 weeks for the combination. No significant differences in survival between the two treatments were apparent for either cell type. Responders lived no longer than nonresponders in this study. Both initial performance status ($P = 0.001$) and extent of disease ($P = 0.01$) were found to be significantly related to survival time.

Crossover Treatment

Only 29 patients (21 with squamous and eight with large cell carcinoma) began secondary treatment with CCNU after progressing during the mechlorethamine-alone treatment. Of the 27 evaluable cases, 15 (56%) received only one dose of CCNU. Two patients experienced life-threatening thrombocytopenia, and both of them had previously experienced severe myelosuppression during mechlorethamine treatment. One patient with large cell carcinoma who had previously experienced tumor regression while receiving mechlorethamine also responded to CCNU. None of the patients with squa-

TABLE 1.—Objective tumor response

Treatment	No. of patients with— Partial regression	No change	Disease progression	Total No. of patients evaluable/total No. nonevaluable
		Squamous cell carcinoma		
Mechlorethamine	6(9%)	33(47%)	31(44%)	70(100%)/6
Mechlorethamine + CCNU	2(3%)	28(41%)	38(56%)	68(100%)/4
		Large cell carcinoma		
Mechlorethamine	3(8%)	20(59%)	16(41%)	39(100%)/2
Mechlorethamine + CCNU	5(11%)	17(37%)	24(52%)	46(100%)/0

mous cell carcinoma experienced objective tumor regression during secondary treatment with CCNU.

DISCUSSION

The therapeutic merits of CCNU in squamous cell and large cell carcinomas of the lung have recently been reviewed by Selawry who found that in 17% of reported cases these two histologic types of cancer have regressed objectively after treatment with CCNU (4). Similarly, composite data from that review indicate that squamous and large cell carcinomas have regressed in 21% and 26% of the patients, respectively, when treated with mechlorethamine as a single agent. In comparison, our results were poor, and the combination regimen was not better than mechlorethamine alone in squamous and large cell carcinoma.

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Sequencing of Cytosine Arabinoside (NSC-63878) and Daunorubicin (NSC-82151) in Acute Myelogenous Leukemia[1,2,3,4]

Brief Reports and Preliminary Communications

George A. Omura[5,6,*]

Daunorubicin (DNR) and cytosine arabinoside (ara-C) are the two most active agents in adult acute myelogenous leukemia (AML) and have been used in combination by several groups. However, the optimal way to combine them has not yet been determined. Edelstein et al (1,2) have previously reported that in their mouse model, the leukemic cell kill, relative to killing of normal hematopoietic cells, was greatest when a single dose of ara-C was given first, followed 10 or 15 hours later by DNR. Giving DNR first was not as effective, and when the two drugs were given simultaneously the cell kill was less than additive. A clinical test of this observation has been made.

MATERIALS AND METHODS

Two schedules of DNR plus ara-C were developed. In the sequence designated AD (ara-C and DNR), a 5-day continuous infusion of ara-C (100 mg/m²/day) was given, followed 24 hours later and in some patients 48 hours later with DNR (100 mg/m²/dose) by rapid iv injection. In the sequence designated DA, DNR (100 mg/m²) was given as a single injection on Day 1 followed immediately by a continuous infusion of ara-C as described above. For either sequence, the course could be repeated after at least a 7-day rest if residual leukemia was evident.

Patients ≥15 years with AML or myeloblastic, myelomonocytic, or promyelocytic leukemia were eligible after written informed consent. Patients who had had prior treatment but who (*a*) were in relapse and had recovered from the toxicity of prior therapy, (*b*) had not relapsed while receiving ara-C, and (*c*) had never received DNR or adriamycin were also eligible. Over a 12-month period, pilot studies of these two sequences were conducted one after the other in a nonrandomized fashion by the same staff. Patients were sequentially selected without the selected deletion of any patient from the study. Table 1 shows the 13 patients (nine women and four men, median age 44 yrs) with AML who received schedule AD and the 11 patients (eight women and three men, median age 46 yrs) who subsequently received schedule DA. Although the groups appear heterogeneous, careful review did not reveal any imbalance which would prejudice the results against the AD sequence. For example, prior treatment might be a disadvantage, but three of five patients in this category responded (CR) on the AD sequence. Moreover, the older patients in the AD group did at least as well as the younger patients or those in the DA group. Remission was defined by the criteria of the Acute Leukemia Group B. Remission duration and survival were not analyzed since a variety of maintenance regimens were used.

RESULTS

Response

All patients completed at least one course of treatment. In addition to comparing remission rates, we noted the time to remission (the quicker the better in order to shorten hospital stay) and the dose of DNR to remission (the lower the better because of cumulative cardiac toxicity from this drug). The superior schedule might show an advantage in one or both of these parameters even if the remission rate was the same.

With the AD sequence, there were five patients whose first course included one dose of DNR; one CR and two PRs resulted. Hoping to improve the response rate, eight subsequent patients received two doses of DNR in the first course, five of whom achieved CR (63%).

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[2]Presented in part to the American Society of Clinical Oncology, San Diego, Calif, May 7, 1975 (abstr 1183).

[3]Supported by Public Health Service grants CA-03013 and CA-13148 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[4]Cytosine arabinoside: CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride.

Daunorubicin: CAS reg. No. 23541-50-6. Supplied by Cancer Therapy Evaluation Program, Division of Cancer Treatment, NCI.

[5]Department of Medicine, University of Alabama, Birmingham.

[6]I wish to thank Drs. J. R. Durant, R. A. Gams, J. T. Carpenter, and C. W. Scott for their help in conducting the clinical trials, and Dr. S.-J. Soong for statistical analysis.

*Reprint requests to: George A. Omura, MD, Department of Medicine, University of Alabama, University Station, Birmingham, Ala 35294.

TABLE 1.—Patient populations*

AD sequence (all AML)					DA sequence (1 dose of DNR/course)				
Age/sex	Prior therapy	No. of courses	Doses of DNR/course	Result	Age/sex	Diagnosis	Prior therapy	No. of courses	Result
17/M	No	2	2,1	CR	17/F	APL	No	1	CR
27/F	No	3	1,1,1	PR	22/F	AML	No	1	CR
30/M	Yes	2	2,1	NR	22/M	APL	No	3	CR
36/F	Yes	1	1	PR	41/F	AML	No	2	DD
40/M	No	3	2,1,1	CR	43/F	AML	No	2	CR
42/F	No	1	2	DD	46/F	AML	No	1	CR
44/F	No	3	1,1,1	DD	52/M	APL	No	2	LD
48/M	Yes	2	2,1	CR	58/F	AML	No	2	CR
51/F	Yes	1	2	CR	60/F	AML	Yes	2	LD
53/F	Yes	1	2	CR	63/F	AML	Yes	2	NR
71/F	No	1	1	LD	68/M	AML	No	2	PR
73/F	No	2	2,2	LD					
74/F	No	1	1	CR					

*CR = complete remission; PR = partial remission; NR = no remission; LD = died from leukemia; DD = died from treatment; and APL = acute promyelocytic leukemia.

Overall, six of 13 patients receiving the AD sequence achieved CR (46%). They required a median dose of 250 mg/m^2 of DNR (range, 100–400 mg/m^2) and a median of 45 days (range, 30–69 days) to achieve CR. Of the 11 patients receiving the DA sequence, there were six CRs (55%) and one PR. The median dose of DNR was 150 mg/m^2 (range, 100–300 mg/m^2) and the median time to CR was 33 days (range, 19–84 days). The differences are not statistically significant (by chi^2 test: $P = 0.68$ for remission rate, $P = 0.62$ for time, $P = 0.22$ for dose). The difference in time to remission is not accounted for by the extra day or 2 required to deliver each course of the AD sequence.

TOXICITY

The hematologic toxicity was similar for the two regimens. None of the patients in either treatment group had a nadir white blood cell count >500 cells/mm^3; no nadir platelet count was >19,000 cells/mm^3.

On the AD schedule, all but one patient developed a hypocellular to aplastic marrow with treatment; there were two drug deaths. On the DA schedule, all patients had a hypocellular to aplastic marrow; there was one drug death on this schedule. Cellularity determinations were made on the basis of bone marrow biopsy specimens. No cardiac toxicity was seen.

DISCUSSION

Gluckman et al (3) achieved a 55% CR rate in AML with an AD sequence using 300 mg/m^2 of DNR per course. They used a concurrent schedule in promyelocytic leukemia in order to have as rapid a response as possible. Concurrent drug administration produced less than additive cell kill in the mouse model but was quite effective (50% CR) in this previously resistant type of human leukemia. Yates et al (4) have used DNR at a dose of 45 mg/m^2 on Days 1–3 with a 7-day infusion of ara-C (100 mg/m^2/day). They reported an overall 65% CR rate and an 87% CR rate in their younger patients. Their median dose of DNR to remission was 160 mg/m^2 (5). Rai et al (6) have had similar results with the 7-day schedule and found that a 5-day course with only two doses of DNR was not as effective.

The results of our study show that AD was of no apparent advantage in achieving remission in adult AML and required more time and more DNR than the DA sequence. The inability to demonstrate a clinical advantage for AD sequences or a disadvantage for the opposite sequence does not necessarily discredit the laboratory model. The mouse model (1,2) used single doses rather than a continuous infusion of ara-C, and no provision was made for giving additional doses of DNR or repeated courses of the combination. The extrapolation from a 10- or 15-hour interval between drug injections in the mouse (1,2) to 24 hours in man may be wrong. Moreover, patients have a heterogeneous group of disorders called AML, in contrast to a relatively homogeneous disease in laboratory animals. Differences in growth fraction in the mouse model and in human leukemia in relapse may help to explain the differing results.

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Phase II Evaluation of VP-16-213 (NSC-141540) and Cytembena (NSC-104801) in Patients With Advanced Breast Cancer[1,2,3]

Brief Reports and Preliminary Communications

David L. Ahmann,* Harry F. Bisel, Robert T. Eagan, John H. Edmonson, Richard G. Hahn, Michael J. O'Connell, and Stephen Frytak[4]

VP-16-213 is a semisynthetic product derived from podophyllotoxin. Studies have indicated that one of its mechanisms of action may be that of a mitotic poison. VP-16-213 appeared to be less toxic than many related compounds which had been derived from podophyllin and has shown an antitumor effect in experimental rodent tumors including the mouse Sarcomas 37 and 180 and the L1210 leukemia system.[5,6] VP-16-213's unique mechanism of action and rather mild toxicity prompted an early clinical trial in patients with advanced breast cancer. The schedules used in this particular clinical trial were derived from a dose-seeking study at this institution, which indicated that from 100 to 125 mg/m^2 could be given safely every other day for a total of three doses every 3–4 weeks with acceptable toxicity.[7]

Cytembena was originally developed by Czechoslovakian investigators. It is an antineoplastic agent resulting in inhibition of purine biosynthesis, inhibition of tetrahydrofolate formylase, and inhibition of incorporation of amino acids including glutamic acid, glycine, and methionine.[8] Cytembena was not active in the L1210 leukemia system but did have cytostatic activity in a number of animal solid tumors including the Sarcoma 180 and mouse mammary carcinoma.[8] Reportedly, it was associated with no hematologic, hepatic, or renal toxicity. However, phase I studies performed at this institution showed that cytembena caused appreciable toxicity when it was given at a dose of > 200 mg/m^2 twice a day for 5 days.[9] The toxic effects observed included a transient rise in serum creatinine, nausea, vomiting, and diarrhea. At the higher dose levels, however, a diffuse autonomic crisis occurred consisting of hypertension, tachycardia, severe vomiting, and uncontrollable diarrhea.[9]

MATERIALS AND METHODS

Thirty-four patients with advanced breast cancer were entered in this randomized clinical trial of VP-16-213 versus cytembena; 19 patients received VP-16-213 and 15 received cytembena. All patients had previously been exposed to multiple-drug chemotherapy and many had had previous hormonal therapy as well. Patients were stratified prior to randomization by dominant disease, free interval, and menstrual status. Patients entered in the study were evenly distributed between VP-16-213 and cytembena, with regard to these discriminating factors. Sixty percent of the patients had visceral-dominant disease and the majority were > 5 years' postmenopausal. Patients were equally divided between those whose free interval was < 1 year and those whose free interval was > 1 year. All of the patients had histologically verified breast cancer with progressive metastatic disease not thought to be amenable to other standard surgical, radiotherapeutic, or chemotherapeutic approaches. All patients had measurable disease which could be serially quantitated and they had an expected survival of > 2 months. All patients had a white blood cell (wbc) count > 4100 cells/mm^3 and a platelet count > 130,000 cells/mm^3. The serum creatinine level was

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[2]Supported by contract N01-CM-53772 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3]VP-16-213: 4′-demethylepipodophyllotoxin 9-(4,6-*O*-ethylidene-β-D-glucopyranoside).

Cytembena: CAS reg. No. 16170–75–5; acrylic acid, 3-*p*-anisoyl-3-bromo-, sodium salt.

Both drugs were supplied by Cancer Therapy Evaluation Program, DCT, NCI.

[4]Division of Medical Oncology, Mayo Clinic and Mayo Foundation, Rochester, Minn.

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**Reprint requests to*: David L. Ahmann, MD, Division of Medical Oncology, Mayo Clinic and Mayo Foundation, Rochester, Minn 55901.

[9]Frytak S, Moertel CG, Schutt AJ, et al. A phase I study of cytembena. Submitted to *Cancer*.

< 1.5 mg/100 ml and the serum bilirubin level was < 2 mg/100 ml. None of the patients had had prior chemotherapy with either of the study agents. Pretreatment assessment and subsequent re-evaluations occurred at 4 weeks and were as previously described in phase II clinical trials in breast cancer (1). An objective response was defined as a measurable decrease of 50% in the product of the two perpendicular diameters of all measurable lesions in the absence of progression of any other lesion or the appearance of new lesions. Patients were classified as having stable disease if there was < 50% improvement, as previously defined, and no progression in any other parameter. If progression occurred, alternate chemotherapy was given on crossover arrangement.

Cytembena was administered at a dose of 200 mg/m² twice a day for 5 days and VP-16-213 was given in 15 of the 19 patients at a dose of 125 mg/m²/day every other day for three doses. One patient received 120 mg/m² and three patients received 100 mg/m² of VP-16-213. The dose of cytembena was not changed on subsequent re-treatment while the dose of VP-16-213 was altered in three patients; two were increased to 150 mg/m² and one was decreased to 100 mg/m².

RESULTS

No therapeutic benefit as defined by an objective decrease in measurable tumor occurred after the use of these two agents in this group of patients. After cytembena, however, five patients had stability of disease with a mean duration of 10 weeks, while seven of the 19 patients had stability of disease following VP-16-213 therapy for a mean of 15.6 weeks prior to the onset of progressive metastatic disease. The mean survival following entry in the study was quite comparable for patients receiving both treatment programs, ie, 12.4 weeks after VP-16-213 therapy and 12.6 weeks after cytembena therapy. Two patients remain alive following treatment with VP-16-213 at 32+ weeks and three patients remain alive following cytembena therapy at 21, 20, and 20 weeks.

Toxic effects were as expected and are shown in table 1. After cytembena administration, the majority of patients experienced mild nausea, vomiting, and diarrhea. These effects, for the most part, were not difficult to control and rarely necessitated the administration of parenteral fluids. Alopecia occurred in a few patients and myelosuppressive effects were not noted. However, wbc and platelet counts, for the most part, were not performed after administration of cytembena since our previous phase I experience revealed the absence of myelosuppression. Renal toxicity as shown by a transient rise in serum creatinine levels occurred in 30% of the patients but serum creatinine was not measured after treatment in many of the patients until re-evaluation. Serum creatinine levels did rise as high as 9.3 mg/100 ml but returned to nearly normal 3–4 weeks after treatment.

TABLE 1.—Toxic effects in patients receiving VP-16-213 and cytembena

Toxic effects	No. of patients with toxic effects/ No. of patients treated	
	VP-16-213	Cytembena
Gastrointestinal	15/28 (53%)	24/25 (96%)
Alopecia	12/28 (42%)	2/25 (8%)
Wbc count ≤ 3000 cells/mm³	18/28 (64%)*	0/25 (0)
Platelet count ≤ 100,000 cells/mm³	4/28 (14%)†	0/25 (0)
Renal	0/28 (0)	7/25 (28%)

*Mean nadir 2200 cells/mm³.
†Mean nadir 66,000 cells/mm³.

VP-16-213 administration was associated with nausea and vomiting in only about half of the patients, with 42% having significant alopecia. Sixty-four percent of the patients had leukocyte depressions of < 3000 cells/mm³ with a mean nadir of 2200 cells/mm³ and a duration of 7–11 days. Fourteen percent of the patients had platelet depression of < 100,000 cells/mm³ with a mean of 66,000 cells/mm³.

DISCUSSION

Neither VP-16-213 nor cytembena in the doses and schedules used in this study was found to have an appreciable antitumor effect as defined as a > 50% reduction of tumor. It must be pointed out, however, that this group of patients had evidence of markedly advanced disease and previously had been treated with a number of cytotoxic chemotherapeutic agents that were usually part of a multiple-drug treatment trial. Significant numbers of patients in both treatment groups did have "stable disease" for a finite period of time and thus some antitumor effect was shown. Previous evaluations of VP-16-213 have, indeed, displayed objective antitumor effect in a comparable group of patients.[10] VP-16-213 is currently being explored in other dose regimens and combinations with other active agents in patients with advanced breast cancer.

[10]*See* footnote 7.

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Yeast Mutants With Distorted Cell Membranes as Tests in the Screening for Antitumor Antibiotics[1]

Brief Reports and Preliminary Communications

G. F. Gause,* A. V. Laiko, and T. I. Selesneva[2]

The isolation of antitumor antibiotics is difficult when cultures of producing microorganisms synthesize complicated mixtures of various natural products. Some of these products may display both antitumor and antibacterial effects and others may display antibacterial action only. In the early phases of such studies, crude concentrates containing mixtures of natural products may show antitumor effect in animal experiments. However, with further purification using various test bacteria, it is easy to disregard the antitumor products and isolate antibacterial substances devoid of any antitumor action. Cultures of cancer cells in vitro used as tests do not always help in the isolation and chemical purification of anticancer products from complex mixtures of natural products.

In view of these observations it is important in the screening for antitumor antibiotics to have a test which possesses high specific sensitivity to anticancer substances and does not react to concomitant natural products with an antibacterial effect. We have recently observed that mutants of yeast cells with distorted cell membranes are of great interest in the screening for natural products with antitumor action.

Common yeast cells are of no great interest as test organisms because they are weakly permeable and not very sensitive to antitumor antibiotics. Prof. F. Lacroute (Laboratory of Physiological Genetics, Institute of Molecular Biology, Strasbourg, France), working with a parent culture of yeast *Saccharomyces cerevisiae* FL 200, recently developed a mutant FL 599–1B which possesses increased sensitivity to antifungal antibiotics.[3] Parent culture FL 200 is inhibited by actidione (cycloheximide) at 30 μg/ml while the mutant FL 599–1B is six times more sensitive and is inhibited by actidione at 5 μg/ml.

Prof. Lacroute very kindly provided us with these cultures and we evaluated both against a limited number of anticancer antibiotics with well-known molecular mechanisms of action. Experiments were made on gradient agar plates with a nutrient medium of the following composition: tryptone 0.3%, sodium chloride 0.5%, peptone 0.5%, glucose 1%, and agar 2%. The results of these studies are shown in table 1.

These results indicate that all antitumor antibiotics tested, with the exception of olivomycin, selectively inhibited the growth of mutant FL 599–1B. Antibiotics with alkylating action on DNA (mitomycin C), antibiotics intercalating DNA (daunorubicin, adriamycin, and carminomycin), a bifunctional intercalating antibiotic (echinomycin), and antibiotics producing single strand breaks in DNA (streptonigrin and bleomycin) all selectively inhibited the culture of mutant FL 599–1B. This selectivity, which took place in spite of differences in the mechanisms of action, indicates that the distorted cell membrane of mutants permits penetration of molecules of these substances into cells of mutants with the resulting inhibitory action. Yet these antibiotics either do not penetrate the cells of the parent culture at all or penetrate to a much smaller degree compared to mutants.

In further studies, the effect of antibacterial antibiotics upon the parent culture of yeast and its mutants was investigated. The results are given in table 2.

TABLE 1.—Effect of anticancer antibiotics on the parent culture of yeast *Saccharomyces cerevisiae* FL 200 and its mutant (FL 599-1B) with distorted cell membranes

Antibiotic	Minimal inhibitory concentration (μg/ml)	
	Parent culture, FL 200	Mutant, FL 599-1B
Actinomycin D	>100	5.0
Mitomycin C	>100	7.0
Daunorubicin	>100	6.0
Adriamycin	>100	5.0
Carminomycin	30	2.0
Streptonigrin	>100	0.3
Bleomycin	5	0.5
Sibiromycin	>100	2.0
Echinomycin	>100	0.7
Olivomycin A	>100	>100

[1]Received Feb 9, 1976; accepted Mar 24, 1976.

[2]Institute of New Antibiotics, USSR Academy of Medical Sciences, Moscow.

[3]FL 599–1B and FL 200 are the European Repository numbers for these cultures.

*Reprint requests to: Prof. G. F. Gause, Institute of New Antibiotics, USSR Academy of Medical Sciences, Bolshaia Pirogovskaia, 11 Moscow, USSR.

TABLE 2.—Effect of antibacterial antibiotics on the parent culture of yeast *Saccharomyces cerevisiae* FL 200 and its mutant (FL 599-1B) with distorted cell membranes

Antibiotic	Minimal inhibitory concentration (μg/ml)	
	Parent culture, FL 200	Mutant, FL 599-1B
Penicillin	>100	>100
Streptomycin	>100	>100
Neomycin	>100	>100
Paromomycin	>100	>100
Lincomycin	>100	>100
Chloramphenicol	>100	>100
Tetracycline	>100	>100
Vancomycin	60	>100

Table 2 shows that the antibacterial antibiotics studied by us did not significantly inhibit the growth of either the mutant or the parent strain. These antibiotics selectively inhibited the synthesis of the cell wall (penicillin and vancomycin) or the synthesis of protein at the ribosome level in the cells of prokaryotes (tetracycline, chloramphenicol, streptomycin, and lincomycin). The cells of eukaryotes, including yeast cells, are not affected by these inhibitors and this is evidently the reason why yeast mutants with distorted cell membranes are not inhibited.

These observations indicate that yeast mutants with distorted cell membranes, which are selectively inhibited by anticancer antibiotics and are not affected by antibacterial antibiotics, could be of considerable interest as test organisms. They could be used for the isolation of active antitumor substances from complex mixtures of natural compounds. This test makes the separation of anticancer products from the accompanying bacterial inhibitors easier and it is presently used at this Institute. It would be desirable to test the utility of this method on a broader range of materials.

LETTER

Serpentine Supravenous 5-Fluorouracil (NSC-19893) Hyperpigmentation[1]

Pigmentary changes have been associated with iv 5-fluorouracil (5-FU) therapy in from 2% to 5% of all patients treated. This hyperpigmentation usually occurs uniformly on sun-exposed parts of the body (1,2). Other distributions of hyperpigmentation have seldom been reported.

A 56-year-old black man with stage D carcinoma of the prostate was treated with weekly 5-FU at a dose of 750 mg/m^2 for 24 consecutive doses. The total dose of 5-FU received was 27 g. A good partial remission was attained. Little hematologic and no gastrointestinal or central nervous system toxicity was experienced. The patient did experience nasal mucosal friability and diffuse hyperpigmentation of the face and hands. Markedly increased pigmentation of the skin immediately overlying the veins used for multiple 5-FU infusions was noted (fig 1). These dark streaks were 1.0–1.5 cm in width and were serpiginous in their course. When a tourniquet was placed around the upper arm it became obvious that each of these hyperpigmented bands overlay a vein into which one or more doses of 5-FU had been infused. The patient habitually took iv injections in the left arm only. The right arm served as a control and was completely free of this lesion. The veins underlying the pigmented streaks were not tender, thrombosed, or sclerosed and were still patent. It is important to note that the patient received no other substance iv during these 24 weeks of 5-FU therapy. The only concurrent medicine was occasional oral Percodan (oxycodone hydrochloride, aspirin, phenacetin, and caffeine).

This phenomenon has been mentioned by Moertel (3). However, a literature search revealed no similar case reports and it is not specified in other large series reporting the toxicities of 5-FU. This report of an interesting and unexpected result of iv 5-FU therapy is presented in order to further its recognition.

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William J. Hrushesky

Cancer Therapy Evaluation Program
Division of Cancer Treatment
National Cancer Institute
Bethesda, Md 20014

March 4, 1976

FIGURE 1.—Hyperpigmented streaks overlying veins used for weekly 5-FU infusions.

[1]5-Fluorouracil: CAS reg. No. 51-21-8.

ANNOUNCEMENTS

Symposium on "Progress, Promise and Controversy in Cancer Chemotherapy"

New York, New York, October 22–23, 1976

This symposium, sponsored by The Chemotherapy Foundation, will be held at the Barbizon Plaza Hotel in New York. National and international panel participants include Gianni Bonadonna, Paul Carbone, Bernard Fisher, James Holland, Lucien Israel, Leonard Price, and others.

For further information and registration contact: Dr. Ezra M. Greenspan, Mount Sinai Medical Center, New York, NY 10029.

Third International Symposium on Platinum Coordination Complexes in Cancer Chemotherapy

Dallas, Texas, October 18–20, 1976

This symposium will be sponsored by the Wadley Institutes of Molecular Medicine in Dallas, Texas. The suggested areas of discussion include: (*a*) Chemistry of Platinum Coordination Complexes and Animal Studies; (*b*) Interactions with Biomacromolecules; (*c*) Bacterial, Viral, and Tissue Culture Studies; and (*d*) Clinical Trials. The deadline for submitting abstracts is June 30, 1976.

For further information please contact: Third International Symposium on Platinum Coordination Complexes in Cancer Chemotherapy, Wadley Institute of Molecular Medicine, 9000 Harry Hines Blvd, Dallas, Tex 75235.

Medical Oncology Fellowships

Medical Oncology Fellowship applications are now being accepted for a 2-year fellowship training program at University Hospital, Boston University Medical Center, Massachusetts. The section has 38 beds and active out-patient clinics, conducts chemotherapy and immunotherapy trials in solid tumors and hematologic malignancies, and is a member of the Eastern Cooperative Oncology Group. Rotations are available to hematology, radiation therapy, and surgical pathology. Opportunity for participation in a major research effort is available within the Cancer Research Center of Boston University Medical Center. One or 2 years of medical residency is a prerequisite.

For further information write: Marc J. Straus, MD, Chief, Section of Medical Oncology, University Hospital, 75 E Newton St, Boston, Mass 02118.

Eighth World Congress of Gynecology and Obstetrics

Mexico City, October 17–22, 1976

The International Federation of Gynecology and Obstetrics announces the Eighth World Congress of Gynecology and Obstetrics to be held October 17–22, 1976, in Mexico City.

Only active members of national federations, societies, or colleges affiliated with the International Federation of Gynecology and Obstetrics may actively participate in this Congress.

There will be five plenary sessions, seminars on current topics of gynecology and obstetrics, correlated seminars, and three special invited lectures. Within this scientific program there will be free communications sessions, films, and precongress courses as well as scientific, technical, commercial, and artistic exhibits. In addition there will be a social program designed to enable attendants to become better acquainted.

The official languages of the Congress will be Spanish, English, and French with simultaneous translation into Spanish, English, French, and German for special lectures and plenary sessions.

Application forms to participate and to enroll in the Congress and further detailed information may be obtained from: Carlos Mac Gregor, MD, General Secretariat, Avenida Baja California 317, Mexico 11, D.F., Mexico.

Award: "Initial Stages of Cancer of the Uterine Cervix"

As a tribute to René Moricard, Associate Professor in Hormonology at the Faculté de Médecine de Paris, his friends have decided to institute a prize of 125.000 Belgian francs for the best experimental work relating to the "Initial Stages of Cancer of the Uterine Cervix." Only original research on this topic will be considered; regretfully, any work which has already been awarded a prize will not be retained.

Manuscripts should be submitted no later than December 1, 1976, to: Dr. R. Vokaer, Clinique Obstétricale et Gynécologique, Hôpital Universitaire Brugmann, Place Van Gehuchten, 4, 1020 Brussels, Belgium.

Medical Oncology Fellowship

A 1- or 2-year fellowship program in Medical Oncology is now available through the Southern Nevada Memorial Hospital in Las Vegas. Fellows will spend 1 year in Las Vegas and 1 year at the University of Arizona (Division of Medical Oncology, Dr. Sidney Salmon, member of the Southwest Oncology Group). One year of internship and 1 year of medical residency (2 years preferred) are the minimum requirements. The stipend is $14,500.

For further details contact: Joseph M. Quagliana, MD, Director of Medical Education, Southern Nevada Memorial Hospital, 2020 W Charleston Blvd, Las Vegas, Nev 89102 (telephone, 702/385–7169).

CORRECTIONS

We regret that the paper "Use of an Elemental Diet in Animals During Treatment With 5-Fluorouracil (NSC-19893)" which appeared in the January issue (Vol. 60, No. 1) contains an error on page 20, table 2. The fourth column, showing the weight changes for control rats, should read as follows:

$+0.32 \pm 0.02$
$+0.31 \pm 0.02$
$+0.30 \pm 0.01$
$+0.31 \pm 0.02$

The authors of the paper "Urinary Excretion of Polyamines by Patients With Advanced Malignancy," which appeared in the November/December issue (Vol. 59, No. 6), regret that several errors appeared with regard to the references. The fifth sentence in the first full paragraph on page 1115 should read as follows: *Lipton et al* (25), using, with minor modifications, the original method employed by Russell (1), reported polyamines were elevated in the urine of approximately two thirds of a group of 56 patients with metastatic disease." Reference 25 is listed incorrectly as Iversen et al and should be as follows:

25. LIPTON A, SHEEHAN LM, and KESSLER GF, JR. Urinary polyamine levels in human cancer. Cancer 35:464–468, 1975.

In the third full paragraph on page 1115, the third sentence should read: "Since Burkitt's lymphoma is a rapidly proliferating tumor with a high growth fraction (*26*), polyamines may also find application in other neoplastic diseases where the rate of cellular multiplication is rapid." Reference 26 should be:

26. IVERSEN U, IVERSEN OH, BLUMING AZ, ET AL. Cell kinetics of African cases of Burkitt lymphoma. A preliminary report. Eur J Cancer 8:305–308, 1972.

The authors and editorial staff regret these errors and apologize to the investigators involved.

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